Using an in vitro model of HIV-1 male-to-female transmission to identify key aspects of the bottleneck hypothesis

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Dedications

I would like to dedicate this thesis to my sanity. You were a wonderful friend and a loving partner. Your time in my head was too short lived. Life will never be the same without you.

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ABSTRACT

Using an in vitro model of HIV-1 male-to-female transmission to identify key aspects of the bottleneck hypothesis

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When semen comes in contact with the female reproductive tract (FRT), it elicits a polarized- and time-dependent release of cytokines and other soluble factors from the epithelial barrier. These factors are involved in tissue remodeling, as well as immune cell activation and recruitment. This influx of immune cells potentially susceptible to human immunodeficiency virus type (HIV-1) infection leads us to hypothesize that (i) semen alters the subepithelial environment of the FRT to be more pro-inflammatory, (ii) the pro-inflammatory environment induced by semen will modulate surface expression of antigens on immune cells, specifically the HIV-1 co-receptors on T cells, and (iii) that the combined effect of semen-induced inflammation and immune cell alterations will increase the risk of male-to-female R5-tropic HIV-1 transmission. To test these hypotheses in vitro, three different cell lines derived from the vagina, ectocervix, and endocervix were used to establish a polarized, transwell-based polarized epithelial model. After introduction of activated PBMCs into the basolateral chamber and apical exposure of the epithelial cells to semen (from healthy donors), the basolateral medium was collected and assessed for cytokine production using an ELISArray of twelve pro-inflammatory cytokines, while the PBMCs were harvested and assayed by flow cytometry for cell surface markers indicative of cell status and susceptibility to HIV-

1 infection. To model HIV-1 transmission, a pseudovirus containing a GFP reporter was constructed in conjunction with either an X4-tropic envelope (HXB2) or an R5-tropic envelope (a known transmitted/founder virus). Immediately after apical semen exposure to the epithelium, PBMCs were collected and infected with a pseudovirus, and then assayed for infection (as indicated by GFP expression). In co-cultures of epithelial cells and PBMCs in the absence of infection, conditioned media in the subepithelial chamber was pro-inflammatory in nature, a condition which was exacerbated in the presence of semen. In three separate co-cultures incorporating each of the three epithelial cell lines, the innate immune response in the absence or presence of semen was dominated by IL-8. Flow cytometric analyses of the PBMCs showed that $CCR5^+$ T lymphocytes decreased in numbers in the absence of semen only in the ectocervical model. However, $CXCR4^+$ T lymphocyte numbers did not change appreciably in any epithelial model system. When PBMCs were pre-conditioned and then infected with an R5 or X4 pseudovirus, only those conditioned by the ectocervical cells displayed increased levels of infection by the R5 virus, but with no change in X4 virus infection. Ongoing studies are investigating the mechanism by which semen preferentially selects for R5 viruses over X4 viruses

Chapter 1

Immunomodulation by seminal factors
and implications for male-to-female HIV-1 transmission

Shawn Keogan, Karl Siegert, Brian Wigdahl, and Fred C. Krebs

1.1 Abstract

The role of semen in heterosexual transmission of the human immunodeficiency virus type 1 (HIV-1) has been marginally viewed as an inert

vehicle for the delivery of virus. However, studies from the field of reproductive biology have made it clear that seminal fluid is a complex and dynamic medium containing high concentrations of factors that play key roles in modulating the local immune response in the female reproductive tract during fertilization and embryogenesis. It is therefore strongly implied that the same seminal factors responsible for guiding the immune response in reproduction also play a role in male-to-female transmission of HIV-1. To begin to understand how these factors affect male-to-female HIV-1 transmission, multiple studies have comparatively profiled the contents of seminal fluid collected from uninfected and HIV-1-infected men. This review provides an overview of these studies, as well as a discussion of the potential impact of semen on HIV-1 transmission.

1.2 Introduction

A growing body of contemporary studies indicates that semen, which in its simplest form is a vehicle for sperm cells, is also an active partner in conception

and an influential and dynamic fluid capable of shaping the ensuing maternal immune response. Studies from the fields of fertility and conception have demonstrated that seminal fluid plays a key role in modulating the immune system of the female reproductive tract (FRT) during conception. Because FRT immune responses (particularly the innate immune response) also play important roles in the defense against sexually transmitted disease (STD) pathogens, it stands to reason that these semen-mediated effects may also influence the transmission of STD pathogens, including the human immunodeficiency virus type 1 (HIV-1).

The effects of semen and its components is an area of study that is of increasing interest to those investigating sexual transmission of HIV-1. In the current acquired immune deficiency syndrome (AIDS) epidemic, 75%-85% of the 34.2 million people living with HIV-1 were infected through sexual contact.[1] Furthermore, it is estimated that 30-40% of those sexually acquired infections were the result of male-to-female transmission,[2] which underscores the importance of understanding all aspects of sexual HIV-1 transmission, including the roles that semen may play in influencing the acquisition of HIV-1 among women at risk for infection.

Studies of HIV-1 transmission have often disregarded the effect of semen during viral infection, assuming that semen was simply an inert vehicle for protecting and transporting sperm cells in the FRT. *In vitro* and *in vivo* experiments focused on HIV-1 transmission are often performed in the absence of semen and, instead, rely on the use of either cell-free virus or infected cells as the source of HIV-1. Experiments that do involve semen are usually focused strictly on its direct

effects on the antiviral efficacies of candidate microbicides. Although experiments such as these are valued for their simplicity and consistency, they fail to account for the biological effects of semen on events that take place during the process of HIV-1 transmission.

Recent studies suggest that semen is a more active participant in the process of HIV-1 infection during sexual intercourse. In a review published in 2011 centered semen and female genital tract secretions, unpublished experiments were cited in which the innate antiviral activity of cervicovaginal lavage fluid decreased in the presence of seminal fluid, indicating that seminal fluid contained factors that were likely modulating responses in the FRT involved in innate immune responses.[3] More recent studies have provided new insights into the effects of semen on the immunological environment within the FRT. Experiments involving FRT-derived epithelial cells exposed to semen suggested that transforming growth factor beta (TGF-β), contained in very high concentrations within the semen, may be responsible for directing a local immune response within the FRT that favors embryo implantation.[4] It was concluded that TGF-β, which is present in all three isoforms in semen, was a major factor responsible for initiating the early local inflammatory response through the induction of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 6 (IL-6) expression from cervical epithelial cells.[4] In addition, previous studies performed in the field of reproductive biology demonstrated that seminal TGF-β was also responsible for inducing a local anti-inflammatory response that favored tolerance of the paternal antigens and embryo implantation during the resolution phase of inflammation.[5]

While these studies strongly suggested that the introduction of semen had a profound effect on the immunological environment within the FRT, they did not reveal the specific effects of semen on HIV-1 transmission.

Investigators who strive to better understand the effects of semen on HIV-1 transmission within the FRT will face at least two challenges. First, semen is a complex mixture of cytokines, chemokines, growth factors, and other biologically active molecules. Some of these factors may serve to promote HIV-1 transmission and infection, while others may have roles as antiviral factors. Other factors may instead be pleiotropic with respect to the risk of HIV-1 transmission, dependent on time following the initiation of coitus and the introduction of semen, regional differences within the FRT, the target cell type, conditions within the FRT associated with concurrent infections, or other physiological conditions. Studies of semen-mediated effects will need to consider this spectrum of activities and strive to assign specific functions in the FRT to individual factors contained within the seminal plasma.

The second challenge will be posed by natural variations in semen content among men. Like studies involving primary tissues or peripheral blood samples, experiments involving semen samples will need to account for natural variations in semen content among men, including changes associated with aging, nutritional status, immunological status, or other pathological conditions in the male reproductive tract (MRT) that may alter the multi-factorial nature of seminal fluid. Adding another layer of complexity to donor-to-donor variability are changes in semen content as a consequence of HIV-1 infection. Several recent studies have

demonstrated significant differences in seminal cytokine and chemokine content between HIV-1-infected and uninfected men.[6, 7] It is also likely that seminal content varies with HIV-1-associated disease progression, administration of antiretroviral therapy, and substance abuse. Therefore, experiments involving semen samples from HIV-1-infected donors will be particularly challenging, requiring multiple donors with detailed clinical histories.

The goal of this review is to provide a starting point for addressing these challenges by summarizing recent investigations that have revealed associations between seminal factor content and HIV-1 infection.

1.3 Semen has a complex composition

1.3.1 Semen composition

Studies of male infertility have provided numerous opportunities to assess the concentrations of biologically active factors contained in semen from both healthy and diseased donors. These studies have clearly shown that semen contains a diverse range of immunomodulatory factors, including cytokines, chemokines, growth factors, hormones, minerals, and ions, that can vary greatly with changes in reproductive health or infection. Changes in the concentrations of these factors can alter the semen profile, resulting in dysregulation, as evidenced by the many types of male infertility that have been identified. These studies have measured various aspects of semen in order to determine correlations between changes in semen content and fertility. However, as mentioned previously, there is a high level of variability across studies due to differences in methodology and

donor diversity, as well as differences in the range of factors measured between studies. Recently, investigations using multiplex technology have provided more comprehensive measures of cytokine levels in semen from uninfected individuals[6-8] [9]. These studies have demonstrated that, in general, seminal fluid has a slightly pro-inflammatory profile, as well as an abundance of hormones and growth factors[8, 9]**.** Additionally, when compared to the peripheral blood compartment, seminal fluid was found to be more concentrated in most measured factors, highlighting the contribution of compartmentalization to the independent development of semen content in the MRT.[6, 10]

In addition to soluble factors and sperm cells, semen also contains cells derived from the male immune system. The average, uninfected male has approximately 5,000 CD4+ T-lymphocytes/ml present in seminal fluid.[11] Macrophages and CD8+ T cells can also be found distributed throughout the MRT, specifically within the epithelium lining the vas deferens and epididymis, and in the interstitial spaces between seminiferous tubules.[12] The CD4+ T-cell population is often elevated in the MRT as a consequence of autoimmunity or STD pathogen infection, and may contribute to the levels of pro-inflammatory factors found in seminal fluid. These changes in content could subsequently lead to an altered immune response once in the FRT.[12]

1.4 Changes in semen content associated with HIV-1 infection

A complete understanding of the effects of semen on male-to-female transmission will require knowledge about the factors that participate in the events

leading to transmission and how those factors vary with HIV-1 infection and relevant clinical variables, such as HIV-1-associated disease progression, current antiretroviral therapy (ART) regimen, substance abuse, and aging. Cytokines, chemokines, and other factors have been shown in several studies to vary significantly in semen from HIV-1-infected men.[6, 8] Additionally, there is also considerable variability in the HIV-1 viral load in semen among infected men.[8, 10]

1.4.1 Changes in biological factor content

Most of the information on the physiological concentrations of factors such as cytokines, chemokines, and growth factors present in seminal fluid has come to light through investigations focused on reproductive biology. These studies typically analyze semen from a group of donors considered to be “normal” relative to other groups subcategorized by infertility diagnoses [9]. Several recent studies, however, have endeavored to build profiles of the contents of semen from HIV-1-positive men as compared to uninfected males (summarized in Table 1.1).

The first major study of this type compared the concentrations of cytokines in semen to the concentrations found in serum.[6] Analyses indicated that semen, independent of HIV-1 infection, had consistently higher concentrations of inflammatory cytokines relative to blood. Additionally, several semen cytokines were further elevated relative to corresponding concentrations in blood from HIV-1-infected men, including IL-1β, IL-4, IL-7, IL-8, GM-CSF, and MCP-1.[6] Elevated concentrations of such factors in the seminal fluid over serum are likely a consequence of MRT compartmentalization.[6] These findings were confirmed in

similar studies comparing semen-to-blood cytokine ratios in HIV-1-infected and uninfected samples; in particular, there was a noted increase in a number of seminal cytokines during HIV-1 infection, including IL-1α, IL-1β, IL-6, IL-7, IL-8, MIP-3α, RANTES, and TGF-β [10, 13, 14].

Table 1.1. Concentrations of factors in human semen change as a result of HIV-1 infection. The table summarizes the results of studies that documented factor concentrations in semen from HIV-1-infected patients or healthy donors. Each entry includes the identity of the factor, changes that occur with HIV-1

infection, and the source references. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon gamma; IL-1α, interleukin 1 alpha; IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-5, interleukin 5; IL-6, interleukin 6; IL-7, interleukin 7; IL-8, interleukin 8; IL-10, interleukin 10; IL-16, interleukin 16; IP10, interferon gamma-induced protein 10; MCP-1, monocyte chemotactic protein 1; MIG, monokine induced by interferon gamma; MIP-1α, macrophage inflammatory protein 1 alpha; MIP-1β, macrophage inflammatory protein 1 beta; MIP-3α, macrophage inflammatory protein 3 alpha; PGE_2, prostaglandin E_2; RANTES, regulated on activation, normal T-cell expressed and secreted; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha.

Table 1.1

Semen component	Changes with HIV-1 infection	Reference
GM-CSF	Studies of GM-CSF in SF were variable; some reported no changes while others observed significant increases. One study observed significant increases specifically during the acute phase of infection	[6, 8, 10]
IFN-γ	Not detected in infected or uninfected SF samples studied	[10, 15]
IL-1α	Increased significantly	[8, 10]
IL-1β	Significantly increased during HIV-1 infection, but decreased significantly after HAART	[6, 10, 15]
IL-2	Not detected in infected or uninfected SF samples studied	[10]
IL-4	Significantly increased	[6]
IL-5	Increased, but not significantly	[6]
IL-6	Significantly increased during HIV-1 infection. Levels were also significantly correlated with changes in semen viral load and stage of infection, with higher concentrations of IL-6 detected during acute phases	[6, 8, 10, 14, 15]
IL-7	Significantly increased	[6, 10]
IL-8	Significantly increased and correlated with elevated semen viral load	[6, 8, 10, 14]
IL-10	No change	[6]
IL-16	Significantly increased	[10]
IP-10	No change	[8]
MCP-1	Significantly increased during HIV-1 infection, but decreased back to uninfected levels during chronic stages of infection	[6, 8, 10]
MIG	Variable findings, ranging from significantly increased to significantly decreased during infection	[6, 10]
MIP-1α	Significantly increased	[8, 10]
MIP-1β	Significantly increased	[8, 10]
MIP-3α	Significantly increased	[10]
PGE2	No change	[8]
RANTES	Significantly increased in HIV-1 positive males. However, significant decreases in RANTES were correlated with elevated semen viral load.	[8, 10, 13]
TGF-β1	Significantly increased in HIV-1 positive males. However, significant decreases in TGF-β were correlated with elevated semen viral load	[8, 10, 15]
TNF-α	Studies on the presence of TNF-α in SF were variable, ranging from undetectable to very low levels in uninfected and infected	[8, 10, 15]

Seminal factor content is also dependent on the stage of HIV-1 infection. A recent study comparing therapy-naïve acute and chronically HIV-1-infected men determined that levels of pro-inflammatory cytokines IL-6, IL-8, and MCP-1 in chronically infected subjects were present at concentrations in semen comparable to those of the uninfected controls. These same factors, however, were significantly increased in the acute phase group.[8] Interestingly, TGF-β was a prominent exception, as there was a three-fold higher concentration in semen from the chronic phase group relative to semen from the acutely infected population.[8] Increases in pro-inflammatory semen cytokines combined with decreases in TGF-β during acute HIV-1 infection suggest that semen from these men might promote a greater risk of infection in the FRT.[8]

1.4.2 Changes in cellular content subsequent to HIV-1 infection and ART

Seminal immune cells, which can contribute to the pool of immunomodulatory factors contained in semen, also vary with HIV-1 infection and antiretroviral therapy. Comparisons of white blood cell (WBC) content in semen revealed that HIV-1-infected men prior to the initiation of ART had fewer WBCs in their semen (1.0×10^5 cells/ml) relative to uninfected men (2.4×10^5 cells/ml).[16] Upon closer inspection of the WBC subpopulations, it was evident that while most HIV-1-infected untreated men maintained a population of macrophages in their semen, CD4+ and CD8+ T cells were not detectable in their semen. In contrast, semen from the average uninfected male contained 5.7×10^3 CD4+ T cells and

1.9 x 10^3 CD8+ T cells per milliliter of semen.[16] Interestingly, upon treatment intervention, all subpopulations of WBCs in infected samples were restored to levels comparable to those of HIV-1-negative subjects.[16] Additional analyses indicated that changes in cell populations were also dependent on the types of antiretroviral drugs included in the patient's regimen. When WBCs were quantified in patients before and after the addition of indinavir (a protease inhibitor capable of penetrating the blood-testes-barrier) to a dual nucleoside ART regimen, the CD4+ T-cell population was further increased in both the peripheral blood and seminal fluid, and increases in CD8+ T cells in the semen were also noted.[16]

Of course, it is important to note that immune cells in HIV-1-infected semen also serve as carriers of the virus in addition to a potential source of immunomodulatory factors. HIV-1 provirus can be isolated from semen-derived macrophages and T cells of HIV-1-positive men, suggesting that both cell types may act as vehicles of cell-associated virus and agents of HIV-1 transmission.[17] Therefore, variations in seminal immune cell content as a consequence of ART will result in changes in the levels of transmissible HIV-1 as well as changes in the contributions that these cells make to the pool of soluble immunomodulatory factors in semen that may impact the risk of HIV-1 transmission.

1.5 Role of changes in biological factor content on HIV-1 transmission and pathogenesis

Factors contained in semen (and changes in their concentration associated with HIV-1 infection and other conditions) have the potential to influence the chain

of events that result in HIV-1 transmission. MCP-1 provides a particularly illustrative example of a biological factor contained in semen that may impact STD pathogen transmission as well as reproductive fitness through variations in concentration. Increased levels of MCP-1 (monocyte chemotactic activating factor, also known as MCAF) may be produced by monocytes, endothelial cells, or fibroblasts present in the MRT and may be involved in protecting the reproductive tract against bacterial pathogens.[18] The role of MCP-1 in reproductive biology, however, is not limited to the male. It has been speculated that once semen has been deposited into the FRT, MCP-1 present in the seminal fluid may be responsible for the local recruitment and activation of monocytes, which may be important in clearing excess sperm.[18] Upon recruitment, these monocytes release IL-6 as a response to MCP-1, resulting in further activation and differentiation into macrophages.[18] The changes in factors initiated by MCP-1 are an example of an intricate cytokine network in which one semen-associated factor is correlated with the expression of other cytokines. In this case, MCP-1-induced IL-6 results in the activation and proliferation of T-lymphocytes, which could play a key role in maintaining or expanding the HIV-1-infected T-lymphocyte founder populations shortly after virus transmission.[19, 20] At the same time, semen IL-8, which is increased in conjunction with MCP-1, may also enhance the role of IL-6 by recruiting additional immune cell populations, including neutrophils and T-lymphocytes that traffic to the FRT.[18, 21]

Controlling this cascade of events as a means of decreasing transmission is an important area of study. A recent study of the sphingosine-1-phosphate (S1P)

receptor modulator FTY720 (Fingolimod), which is being considered for use as a topical vaginal microbicide, demonstrated inhibition of infection in the genital tract of macaques by decreasing the levels of pro-inflammatory mediators IL-8 and IL-1Rα and reducing the number of CD4+ T cells in the genital tract.[21] These findings highlight the potential contribution of elevated semen-derived IL-8 on HIV-1 infection in the FRT, which may cause increased peripheral CD4+ T-cell trafficking and activation of subepithelial T cells in the local environment, leading to an increased presence of immune target cell populations and greater likelihood of local HIV-1 infection. This study also provides an excellent example of preventative approaches that indirectly target events within the FRT that impact HIV-1 transmission instead of directly targeting HIV-1 infection.

HIV-1-infected seminal fluid is also enriched in other factors that may enhance target cell recruitment. For example, increases in macrophage inflammatory protein (MIP)-1α and -1β, as well as regulated and normal T-cell expressed and secreted protein (RANTES), could induce the local recruitment and activation of macrophages, dendritic cells, and T cells within the subepithelium of the FRT. Similarly, the growth factor granulocyte macrophage colony stimulating factor (GM-CSF) induces the maturation of dendritic cells and macrophages at the site of deposition.[6, 8, 22] Once local cells are infected, GM-CSF may boost viral replication in target cells through upregulation of HIV-1 long terminal repeat (LTR) activation and viral transcription.[22, 23]

Seminal fluid from HIV-1 infected males also contains increased concentrations of pro-inflammatory cytokines,[24] which may play a large role in

increasing the likelihood of transmission in the FRT. Studies of FRT tissue indicate that the majority of T cells in the cervicovaginal tract have an effector memory phenotype, a population that upon activation was more susceptible to infection by R5 HIV-1 (a virus that uses CCR5 as its co-receptor) relative to X4 HIV-1, making this cell population a prime target for transmitted HIV-1.[25] Activation of the susceptible resident immune cells may be induced by the inflammatory cytokines present in seminal fluid. However, in addition to the direct effects on T-cell infection kinetics, inflammatory cytokines in semen may also be responsible for inducing increases in permeability of the epithelial barrier lining the FRT. For example, IFN-γ has been shown to enhance permeability by inducing the internalization of tight junction proteins.[26] Similarly, intestinal epithelial monolayers become more permeable with IL-1β exposure via NF-κB activation.[27] The resulting increases in permeability may be key to enhancing the ability of HIV-1 to cross the epithelial barrier, providing direct access to resident subepithelial target immune cells.[28] Increases in epithelial permeability may also facilitate the diffusion of seminal immunomodulatory factors through the epithelial barrier to subepithelial immune cell populations. Cervicovaginal epithelial cells can also serve as intermediaries, releasing immunomodulatory factors in a basolateral direction in response to apical exposure to soluble factors in semen. Subsequent to the introduction of HIV-1-infected semen into the FRT, both mechanisms may contribute to changes in the subepithelial microenvironment and effects on local immune cell populations.

Of particular relevance to HIV-1 transmission are the effects of TGF-β. TGF-β, as described previously, is found in very high concentrations within seminal fluid,

but its concentration varies depending on the state of HIV-1 disease.[8] In acutely infected males, there is a lower concentration of TGF-β and higher levels of pro-inflammatory factors as compared to chronically infected and uninfected individuals. This cytokine profile may favor viral replication via LTR activation, since higher TGF-β levels in seminal fluid of chronically infected males were associated with HIV-1 suppression.[8] Of the three isoforms of TGF-β present in seminal fluid, TGF-β3 was detected in the highest concentrations and has been shown to induce the release of GM-CSF and IL-6 in the FRT.[4] Combined with the findings already discussed on GM-CSF and IL-6, it is likely that the effects of TGF-β on cytokine release are important to HIV-1 transmission. During exposure to the seminal fluid of an acutely infected male, TGF-β will induce the release of GM-CSF within the FRT, leading to the recruitment of target cell populations. These cells may become activated in the pro-inflammatory environment and become more susceptible to infection. After infection, the low ratio of TGF-β to pro-inflammatory cytokines (such as IL-6) in the semen may result in increased viral replication within infected immune cells.

It is clear that the factors in seminal fluid are part of a complex network of interactions. A recent study of 21 analytes in seminal fluid identified 72 statistical correlations that exist between cytokines, chemokines, and growth factors in connection with either HIV-1 load or $CD4^+$ T-cell count. Furthermore, changes in individual factors were also interrelated to changes in other seminal cytokines[24] The findings in this study illustrate the complexity of the semen cytokine network. For example, HIV-1 infection was correlated with increased levels of pro-

inflammatory cytokines, including IL-1α, IL-1β, IL-6R, and RANTES in seminal fluid.[24] RANTES was positively correlated with increases in MIP-3α, which in turn corresponded to increases in IL-8 in semen. Furthermore, levels of IL-8 were associated with increased expression of IL-6, MIP-1β, and MCP-1, which together provide pro-inflammatory feedback. Establishing a concrete relationship between changes in the factors that originate in the MRT and the presence of HIV-1 in semen will be important to developing an understanding of their influence on virus transmission within the FRT.

1.6 Summary

Semen is a dynamic fluid capable of orchestrating and influencing immune responses within the FRT. The numerous agents in semen that profoundly affect the immunological environment of the FRT include cytokines, chemokines, growth factors, and other biologically active molecules. Changes in these factors brought about by HIV-1 infection or other influences may affect key events that take place during male-to-female HIV-1 transmission, resulting in an increased or decreased risk of transmission. While studies have provided useful glimpses into factors in semen that change in concentration subsequent to HIV-1 infection, many questions remain to be answered about changes that occur in the semen as a consequence of HIV-1 infection and related variables (Fig. 1.1A). Once answers to these questions have been obtained, subsequent queries will explore mechanisms of HIV-1 transmission within the FRT that are affected by changes in the content of immunomodulatory factors in semen (Fig. 1.1B). Having greater

knowledge about the dynamics of biological factors in semen will increase our understanding of HIV-1 transmission and provide insights into new opportunities for preventing HIV-1 transmission in women at risk for infection in the current epidemic.

FIGURE 1.1

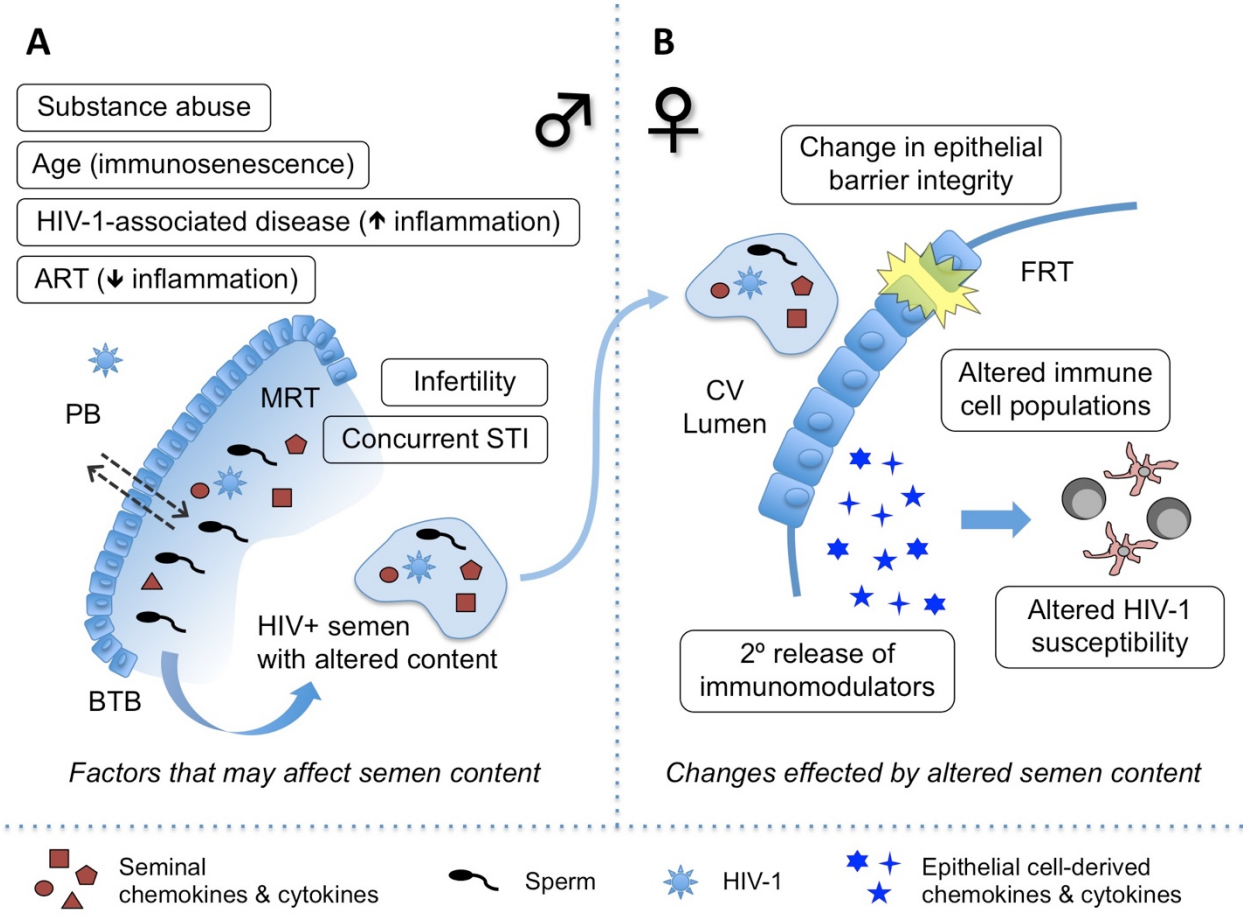
A
B
♂
♀
Substance abuse
Age (immunosenescence)
HIV-1-associated disease (↑ inflammation)
ART (↓ inflammation)
PB
MRT
Infertility
Concurrent STI
HIV+ semen
with altered content
BTB
Change in epithelial
barrier integrity
FRT
CV
Lumen
Altered immune
cell populations
Altered HIV-1
susceptibility
2º release of
immunomodulators
Factors that may affect semen content
Changes effected by altered semen content
Seminal
chemokines & cytokines
Sperm
HIV-1
Epithelial cell-derived
chemokines & cytokines

FIG. 1.1. Factors in the male reproductive tract (MRT) that can affect semen content and the effects of those changes on epithelial and immune cells in the female reproductive tract (FRT). (**A**) Clinical variables (substance abuse, age, HIV-1-associated disease, and ART) may influence the content of semen through the BTB. MRT pathological conditions (infertility, concurrent STI) will directly affect the semen content. The result will be the production of semen with altered content of immunomodulatory factors. (**B**) Semen with altered immunomodulator content will directly affect CV epithelial cells and indirectly impact subepithelial immune cells through the stimulated, polarized release of secondary factors from semen-exposed epithelial cells. ART, antiretroviral therapy; BTB, blood-testes barrier; CV, cervicovaginal; PB, peripheral blood; STI, sexually transmitted infection.

Chapter 2

Variability in human semen content and its potential effects in the female reproductive tract

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2.1 Abstract

Human semen is a complex medium containing high concentrations of cytokines, chemokines, and growth factors that play key roles in orchestrating immune responses during reproduction. These factors are essential to establishing conditions that facilitate fertilization and embryogenesis through modulation of local immune responses in the female reproductive tract. Typically, semen initiates a biphasic process of inflammation that is gradually resolved, leading to immune cell recruitment pivotal to clearing excess sperm and establishing tolerance of the fetal allograft. However, the identity and concentration of factors found in semen may be altered in the male reproductive tract as a consequence of sexually transmitted infections and infertility conditions. As a result, imbalances in semen content can skew the secretory response of the cervicovaginal epithelium after deposition during heterosexual intercourse, which may distort local immune activity and lead to embryo rejection or enhanced pathogen transmission. Recognizing the array of factors contained in semen and the degree to which they vary is an essential part of understanding the impact of variations in semen content on reproductive biology and the transmission of sexually transmitted disease pathogens.

2.2 Introduction

There is a growing body of reproductive biology information that indicates a more active role for semen in the female reproductive tract (FRT) than initially suspected. A review of semen and female genital tract secretions in the context of microbicide development cited unpublished experiments that suggested the presence of factors in seminal fluid that were likely modulating innate immune responses in the FRT [3]. More recent studies involving FRT-derived epithelial cells exposed to semen suggested that transforming growth factor beta (TGF-β), present in very high concentrations within semen, may be responsible for directing a local immune response within the FRT that favors embryo implantation [4]. It was concluded that TGF-β was a major factor responsible for initiating the early, local inflammatory response through the induction of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 6 (IL-6) expression from cervical epithelial cells [4] [29]. Additional studies demonstrated that seminal TGF-β was also responsible for inducing a local anti-inflammatory response that favored tolerance of the paternal antigens and embryo implantation during the resolution phase of inflammation [5]. These studies strongly suggest that the introduction of semen has a profound effect on the immunological environment within the FRT, which may influence both reproduction and heterosexual disease transmission.

Gaining an understanding of the effects of semen within the FRT is confounded by multiple factors, not the least of which is the complexity presented by the involvement of two separate systems – the male reproductive tract (MRT) and the FRT – each with their own anatomical and immunological aspects to

consider. Semen is a complex mixture of cytokines, chemokines, and biologically active molecules. The concentration of these factors is influenced by donor-specific variables at multiple levels (health, diet, age, infection, and substance abuse) and can be further altered as a consequence of infertility. Studies have demonstrated significant differences in seminal cytokine and chemokine content between infertile and fertile men, which can vary depending on the type of infertility condition and disease progression. The activities of these factors once deposited in the FRT add another level of variability. The FRT, in turn, is subject to its own unique set of variables, including hormones, age, and conditions associated with concurrent infections or other physiological conditions [29]. Studies of semen-mediated effects will need to identify which responses are separately controlled by female or male factors, and which outcomes result from combined interactions.

The goal of this review is to document the variability of semen and semen-derived factors and explore their roles in initiating the local immune responses within the FRT that are key to successful reproduction and prevention of infection.

2.3 Semen has a complex and variable composition

Mature sperm cells in the testes that are ready for ejaculation pass through the epididymis where prostate fluid, which is rich in proteins and other factors such as citric acid and zinc [30], is added (Fig. 2.1). Prior to release, semen is further augmented by fluid from the seminal vesicles, which are responsible for establishing the alkaline pH of semen [30]. The average volume of seminal fluid ejaculated is 3.5 ml, but can range anywhere from 100 µl to 11 ml [31]. Just as the

total volume varies, the concentrations of biologically active factors, including hormones, mucin, cadaverine, putrescine, spermine, enzymes, cytokines/chemokines, fructose, and vitamin D, and other soluble factors present in seminal fluid [32], can also vary significantly not only between donors but also within one individual over time. Semen content can also be affected by other factors, such as changes in fertility, age, frequency of ejaculation, infection by sexually transmitted disease (STD) pathogens, substance abuse, prescription medications, and other factors that reflect an individual's lifestyle [33, 34]. To address the elements of intra- and inter-donor variability, experiments requiring semen have either used large cohorts or often incorporated combined seminal fluid samples from five or more donors, creating working samples with greater volume and content more representative of the "average" male semen content.

Studies of male infertility have provided numerous opportunities to assess the concentrations of biologically active factors contained in semen. These studies have clearly shown that (i) semen contains a diverse and wide range of immunomodulatory factors that likely participate in reproduction as well as pathogen defense, and (ii) the concentrations of these factors can vary greatly with changes in reproductive health or infection. Table 2.1 is a compilation of biological factors detected in semen across numerous studies along with their concentrations, clinical conditions under which they were collected, and the degree to which their concentrations varied with MRT pathology. Some of these factors are highlighted in this review.

Figure 2.1

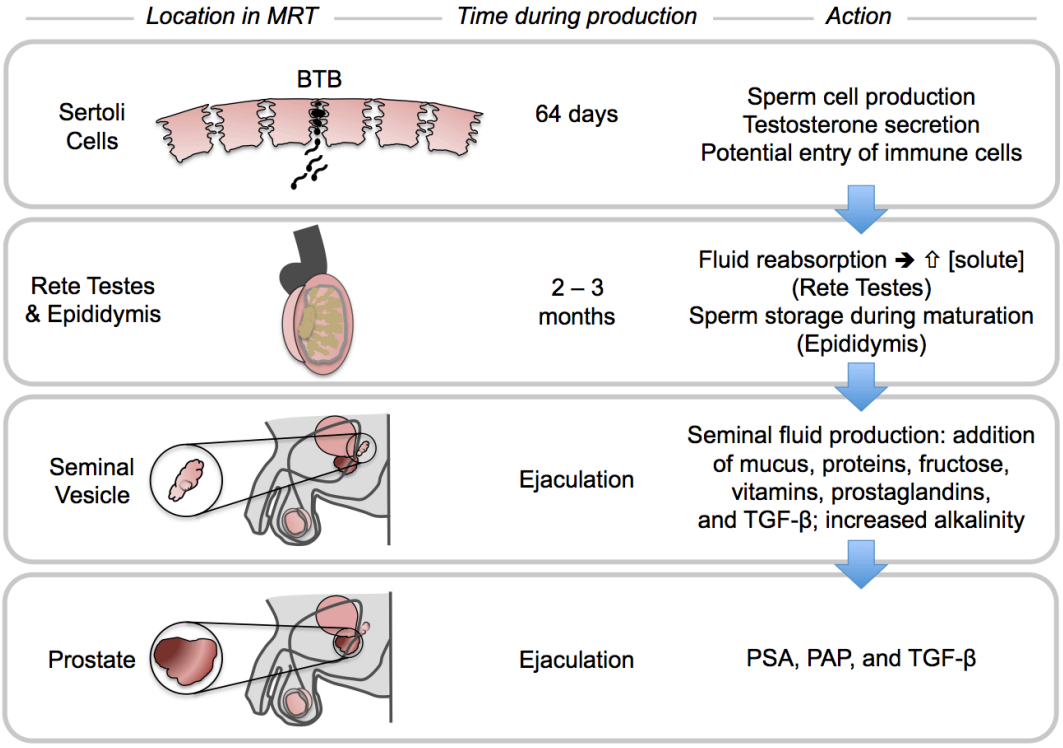

Fig. 2.1. Flow diagram of semen production within the human male reproductive tract (MRT). This schematic representation of the MRT illustrates the tissues involved in semen production within the MRT, the timing of semen production, and the points at which seminal fluid volume and content are altered. BTB, blood-testes barrier; PSA, prostate-specific antigen; PAP, prostatic acid phosphatase; TGF-β, transforming growth factor beta.

Table 2.1 Human semen contains numerous biologically active factors that change with MRT pathology. The table summarizes the results of studies focused on semen content. (A) Physical factors and proteins. (B) Pro-inflammatory cytokines. (C) Anti-inflammatory cytokines. (D) Chemokines. (E) Growth and signaling factors. Each entry includes the identity of the factor, donor health or associated condition, demonstrated concentration (or concentration range), and the reference cited. Concentration ranges for factors from men who were healthy or diagnosed with a clinical abnormality ("abnormal") were compiled (shown in **bold**) if multiple studies of a particular factor were available. Abbreviations: ND, not detectable; E2, estradiol; FSH, follicular stimulating hormone; PAP, prostatic acid phosphatase; LH, luteinizing hormone; GM-CSF, granulocyte-macrophage colony-stimulating factor; PRL, prolactin; IFN-γ, interferon gamma; IL-1α, interleukin 1 alpha; IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-5, interleukin 5; IL-6, interleukin 6; IL-7, interleukin 7; IL-8, interleukin 8; IL-10, interleukin 10; IL-12, Interleukin 12; IL-16, interleukin 16; IL-17, Interleukin 17; IL-18, Interleukin 18; IL-23, interleukin 23; IP10, interferon gamma-induced protein 10; I-TAC, inducible T-cell alpha chemoattractant; MCP-1, monocyte chemotactic protein 1; MIF, macrophage migration inhibitory factor; MIG, monokine induced by interferon gamma; MIP-1α, macrophage inflammatory protein 1 alpha; MIP-1β, macrophage inflammatory protein 1 beta; MIP-3α, macrophage inflammatory protein 3 alpha; PGE_2, prostaglandin E_2; RANTES, regulated on activation, normal T-cell expressed and secreted; SDF-1α, stromal derived factor 1; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha.

Table 2.1A

Cytokine/ Chemokine/ Factor	State of fertility/ disease	Concentration		Reference
Sperm Count				
	Healthy	***27.1 x 10^6/ml – 110 x 10^6/ml***		
	Abnormal	***0 – 98.9 x 10^6/ml***		
	Healthy	Healthy	42.4 x 10^6/ml 110.4 x 10^6/ml 84 x 10^6/ml 49.3 x 10^6/ml 72.98 x 10^6/ml 27.1 x 10^6/ml 79 x 10^6/ml 58.07 x 10^6/ml 64.4 x 10^6/ml 87.6 x 10^6/ml 76.7 x 10^6/ml 77.5 x 10^6/ml 90 x 10^6/ml	[1, 35-46]
		Fertile (smoker)	77.6 x 10^6/ml	[43]
		Fertile + infection	32.9 x 10^6/ml	[35]
		20 – 25 y old males	76.3 x 10^6/ml	[47]
		50 – 55 y old males	73.1 x 10^6/ml	[47]
	Infertile	Infertile (no pathogen infection)	37 x 10^6/ml, 38.3 x 10^6/ml 3.7 x 10^6/ml	[43, 44] [37]
		Infertile + pathogen infection	25 x 10^6/ml	[37]
		Infertile (smoker)	31.5 x 10^6/ml	[43]
		Normospermia	98.9 x 10^6/ml 54.04 x 10^6/ml 56.10 x 10^6/ml	[40, 41, 48]
		Leukospermia	26.3 x 10^6/ml	[35]
		Leukospermia + infection	20.5 x 10^6/ml	[35]
		Oligozoospermia	7.1 x 10^6/ml 6.8 x 10^6/ml 9.78 x 10^6/ml 8.31 x 10^6/ml	[38, 41, 48]
		Azoospermia	0	[36]
		Asthenozoospermia	44.3 x 10^6/ml 43.49 x 10^6/ml 54.55 x 10^6/ml, 45.3 x 10^6/ml	[38, 40, 41, 46]

		Oligoasthenozoospermia	8.3 x 10^6/ml	[46]
		Oligo-terato-asthenozoospermia	4.5 x 10^6/ml	[38]
		Oligo-terato-asthenozoospermia + varicocele	16.5 x 10^6/ml	[36]
		Oligo-terato-asthenozoospermia + genital tract infection	14.2 x 10^6/ml	[36]
		Oligo-terato-asthenozoospermia + testicular lesion	12.8 x 10^6/ml	[36]
Adrenaline				
	Healthy		7.60 ng/ml	[41]
	Infertile	Normozoospermia	3.12 ng/ml	[41]
		Oligozoospermia	2.85 ng/ml	[41]
		Asthenozoospermia	2.71 ng/ml	[41]
Chloride				
	Healthy		1.42 mg/ml	[32]
Citrate				
	Healthy		528 mg/100ml	[32]
Dopamine				
	Healthy		4.34 ng/ml	[41]
	Infertile	Normozoospermia	2.36 ng/ml	[41]
		Oligozoospermia	2.36 ng/ml	[41]
		Asthenozoospermia	1.82 ng/ml	[41]
E2				
	Infertile	Normospermia	25.1 ng/ml	[48]
		Oligozoospermia	24.8 ng/ml	[48]
		Azoospermia	22.5 ng/ml	[48]
FSH				
	Healthy	***2.4 – 7.2 mIU/ml***		
	Abnormal	***2.4 – 10.2 mIU/ml; 4.1 – 19.9 ng/ml***		
	Healthy		2.4 mIU/ml 5.67 mIU/ml 6.9 mIU/ml 7.2 mIU/ml 7.1 mIU/ml 4.4 mIU/ml	[38, 40, 49-52]
	Infertile		10.2 mIU/ml	[50]
		HCV+	2.4 mIU/ml	[52]
		Infertile (spinal cord injury)	2.8 mIU/ml	[50]
		Normospermia	4.1 ng/ml 6.07 mIU/ml	[40, 48]
		Oligozoospermia	8.8 ng/ml 7.78 mIU/ml 6.8 mIU/ml	[38, 40, 48]
		Asthenozoospermia	6.49 mIU/ml 7.3 mIU/ml	[38, 40]
		Oligoasthenozoospermia	9.5 mIU/ml	[38]
		Azoospermia	19.9 ng/ml	[48]
Fructose				

	Healthy	***1.5 – 3.63 mg/ml***		
	Abnormal	***2.18 – 2.49 mg/ml***		
	Healthy		2.72mg/ml 3.63 mg/ml	[32, 40]
		20 – 25 y old males	1.89 mg/ml	[47]
		50 – 55 y old males	1.5 mg/ml	[47]
	Infertile	Normospermia	2.49 mg/ml	[40]
		Oligozoospermia	2.18 mg/ml	[40]
		Asthenozoospermia	2.32 mg/ml	[40]
Glucose				
	Healthy		102 mg/ml (highly variable)	[32]
LH				
	Healthy	***1.53 – 7.94 mIU/ml***		
	Abnormal	***3.1 – 6.87 mIU/ml; 2.9 – 7.3 ng/ml***		
	Healthy		1.53 mIU/ml 6.58 mIU/ml 7.94 mIU/ml 5.5 mIU/ml 6.2 mIU/ml 6.2 mIU/ml 4.7 mIU/ml	[38-40, 49-52]
	Infertile		3.5 mIU/ml	[50]
		HCV+	2.8 mIU/ml	[51]
		Infertile (spinal cord injury)	3.1 mIU/ml	[50]
		Normosperimia	2.9 ng/ml 6.87 mIU/ml	[40, 48]
		Azoospermia	7.3 ng/ml	[48]
		Oligoasthenozoospermia	6.0 mIU/ml	[38]
		Asthenozoospermia	3.82 mIU/ml 6.1 mIU/ml	[38, 40]
		Oligozoospermia	5.1 ng/ml 4.02 mIU/ml 5.9 mIU/ml	[38, 40, 48]
Magnesium				
	Healthy		0.11 mg/ml, 0.07 mg/ml	[32{Colagar, 2009 #85] [43]
		Fertile (smoker)	0.06 mg/ml	[43]
	Infertile		0.06 mg/ml	[43]
		Infertile (smoker)	0.05 mg/ml	[43]
Noradrenaline				
	Healthy		9.29 ng/ml	[41]
	Infertile	Normozoospermia	5.41 ng/ml	[41]
		Oligozoospermia	4.12 ng/ml	[41]
		Asthenozoospermia	3.44 ng/ml	[41]
PAP				
	Healthy	20 – 25 y old males	608 IU/ml	[47]

		50 – 55 y old males	497 IU/ml	
PGE2				
	Healthy		19.67 µg/ml 0.016 µg/ml	[8, 53]
	Infertile	Oligoterato-asthenoazoospermia + genital infection	7.67 µg/ml	[53]
Potassium				
	Healthy		1.09 mg/ml, 0.78 mg/ml	[32, 43]
		Fertile (smoker)	0.74 mg/ml	[43]
	Infertile		0.74 mg/ml	[43]
		Infertile (smoker)	0.67 mg/ml	[43]
PRL				
	Healthy	***7.10 – 290 ng/ml; 320 – 344 pmol/L***		
	Abnormal	***6.5 – 10.57 ng/ml; 110 – 384 pmol/L***		
	Fertile		290 ng/ml 7.10 ng/ml 320 pmol/L 352 pmol/L 344 pmol/L	[39, 40, 50-52]
	Infertile		110 pmol/L	[50]
		HCV+	384 pmol/L	[51]
		Infertile (spinal cord injury)	362 pmol/L	[50]
		Normospermia	8.2 ng/ml 7.21 ng/ml	[40, 48]
		Asthnozoospermia	7.78 ng/ml	[40]
		Azoospermia	6.5 ng/ml	[48]
		Oligozoospermia	8.8 ng/ml 10.57 ng/ml	[38, 48]
Putrescine				
	Healthy	20 – 25 y old males	113 µM	[47]
		50 – 55 y old males	32.3 µM	
Sodium				
	Healthy		3 mg/ml, 2.2 mg/ml	[32, 43]
		Fertile (smoker)	2.1 mg/ml	[43]
	Infertile		2.1 mg/ml	[43]
		Infertile (smoker)	2.1 mg/ml	[43]
Sodium Citrate				
	Healthy		5.28 mg/ml	[32]
Spermidine				
	Healthy	20 – 25 y old males	251 µM	[47]
		50 – 55 y old males	256 µM	
Spermine				

	Healthy	20 – 25 y old males	2.29 mM	[47]
		50 – 55 y old males	2.20 mM	
Testosterone				
	Healthy	***6.6 – 17.73 ng/ml; 0.98 – 17.2 nM***		
	Abnormal	***3.51 – 5.80 ng/ml; 11.6 – 20.4 nM***		
	Healthy		6.6 ng/ml 17.73 pg/ml 7.09 ng/ml 16.3 nM 17.2 nM 17.7 nM 16.2 nM	[38-40, 49-52]
		20 – 25 y old males	0.98 nM	[47]
		50 – 55 y old males	0.99 nM	
	Infertile		11.6 nM	[50]
		HCV+	7.8 nM	[51]
		Infertile (spinal cord injury)	12.1 nM	[50]
		Normospermia	4.1 ng/ml 5.80 ng/ml	[40, 48]
		Asthenozoospermia	4.32 ng/ml 20.4 nM	[38, 40]
		Azoospermia	4.7 ng/ml	[48]
		Oligoasthenozoospermia	15.2 nM	[38]
		Oligozoospermia	4.6 ng/ml 3.51 ng/ml 16.6 nM	[38, 40, 48]
Vitamin A				
	Healthy		28.61 µg/dL	[40]
	Infertile	Normozoospermia	17.86 µg/dL	[40]
		Oligozoospermia	16.86 µg/dL	[40]
		Asthenozoospermia	15.23 µg/dL	[40]
Vitamin C				
	Healthy		5.64 mg/dL	[40]
	Infertile	Normozoospermia	4.18 mg/dL	[40]
		Oligozoospermia	4.95 mg/dL	[40]
		Asthenozoospermia	5.05 mg/dL	[40]
Vitamin E				
	Healthy		0.143 mg/dL	[40]
	Infertile	Normozoospermia	0.109 mg/dL	[40]
		Oligozoospermia	0.089 mg/dL	[40]
		Asthenozoospermia	0.078 mg/dL	[40]
Zinc				
	Healthy		0.17 mg/ml 0.14 mg/ml	[32, 43]
		20 – 25 y old males	2.41 mM	[47]
		50 – 55 y old males	3.12 mM	[47]
		Fertile (smoker)	0.12 mg/ml	[43]
	Infertile		0.10 mg/ml	[43]

		Infertile (smoker)	0.08 mg/ml	[43]

Table 2.1B

Cytokine/ Chemokine/ Factor	State of fertility/ disease	Concentration		Reference
IFN-α				
	Healthy		15.6 pg/ml	[42]
	Infertile	Normozoospermia	92.0 ng/ml	[48]
		Azoospermia	132.6 ng/ml	[48]
		Oligospermia	162.4 ng/ml	[48]
IFN-γ				
	Healthy	***ND – 79 pg/ml; ND – 0.31 IU/ml***		
	Abnormal	***26.8 – 4900 pg/ml; 0.33 – 0.43 IU/ml***		
	Healthy		ND ND 28.7 pg/ml 45 pg/ml 0.25 IU/ml 0.31 IU/ml 3.7 pg/ml 32.2 pg/ml 3.68 fmol/ml 79 pg/ml	[10, 15, 36, 42, 44, 54-56]
		+ *Schistosoma haematobium* infection (low seminal egg secretion)	21.4 pg/ml	[54]
		+ *Schistosoma haematobium* infection (high seminal egg secretion)	120.6 pg/ml	[54]
	Infertile	Infertile	0.33 IU/ml, 6.36 fmol/ml	[44, 56]
		Immunoinfertile (sperm abs)	0.43 IU/ml	[56]
		Normospermia	3600 pg/ml 1.62 pg/ml	[48, 57]
		Genital infection	30.5 pg/ml	[36]
		Varicocele	28.2 pg/ml	[36]
		Cryptorchidism	29.8 pg/ml	[36]
		Mumps orchitis	26.8 pg/ml	[36]
		Idiopathic testicular lesion	28.3 pg/ml	[36]
		Klinefelter syndrome	28.3 pg/ml	[36]
		Oligozoospermia	4900 pg/ml 608 pg/ml	[48, 55]
		Azoospermia	11600 ng/ml	[48]
		Azoospermic obstructive	1049.5 pg/ml	[55]
		Azoospermic non-obstructive	901 pg/ml	[55]
		Ashthenospermia	563 pg/ml 1.88 pg/ml	[55, 57]
		Oligoasthenospermia	629 pg/ml	[55, 57]

			1.2 pg/ml	
IL-1α				
	Healthy		150 pg/ml 4.67 pg/ml 4.7 pg/ml	[8, 42]
IL-1β				
	Healthy	***ND – 19 pg/ml***		
	Abnormal	***ND – 59.3 pg/ml***		
	Healthy		7.97 pg/ml 19 pg/ml 5.0 pg/ml 4.9 pg/ml ND 8 pg/ml 2.3 pg/ml 14.7 pg/ml	[15, 42, 49, 56, 58-61]
	Infertile	Infertile	11.9 pg/ml 7.0 pg/ml ND 37.8 pg/ml	[49, 56, 59]
		Normozoospermia	2.8 pg/ml 0.41 pg/ml	[57, 62]
		Normozoospermia + infection	3 pg/ml	[61]
		Leukocytospermia	26 pg/ml	[60]
		Oligozoospermia	1.5 pg/ml	[62]
		Oligoasthenospermia	0.76 pg/ml	[57]
		Asthenospermia	0.37 pg/ml	[57]
		Immunoinfertile (sperm abs)	ND	[56]
		Infertile + genital infection	59.3 pg/ml 11.7 pg/ml	[49, 61]
IL-6				
	Healthy	***3 – 118 pg/ml***		
	Infertile	***12.4 – 272 pg/ml***		
	Healthy		6.94 pg/ml 10.8 pg/ml 118 pg/ml 18 pg/ml 19 pg/ml 23.84 pg/ml 21.5 pg/ml 23.6 pg/ml 27.7 pg/ml 6.2 pg/ml 3 pg/ml 50 pg/ml 6.4 pg/ml 42.2 pg/ml 25.3 pg/ml 36 pg/ml 31.6 pg/ml	[8, 15, 18, 36, 42, 44, 45, 49, 54-56, 58-61, 63]

		+ *Schistosoma haematobium* infection (low seminal egg secretion)	100.2 pg/ml	[54]
		+ *Schistosoma haematobium* infection (high seminal egg secretion)	591.4 pg/ml	[54]
	Infertile	Infertile	22.9 pg/ml 63.1 pg/ml 46 pg/ml 34.4 pg/ml	[44, 49, 56, 59]
		Normozoospermia	18 pg/ml 29.61 pg/ml	[57, 62]
		Normozoospermia + infection	244 pg/ml	[61]
		Immunoinfertile (sperm abs)	40.5 pg/ml	[56]
		Infertile + genital infection	272 pg/ml 196 pg/ml	[49, 61]
		Azoospermic obstructive	42 pg/ml	[55]
		Azoospermic non-obstructive	37 pg/ml	[55]
		Leukospermia	21.05 pg/ml 15 pg/ml	[18, 60]
		Non-leukospermia	3.24 pg/ml	[18]
		Ashthenospermia	69.5 pg/ml	[55]
		Oligozoospermic	38 pg/ml 40.62 pg/ml 25 pg/ml	[55, 62, 63]
		Oligoasthenospermia	31 pg/ml	[55, 57]
		Asthenospermia	40.09 pg/ml	[57]
		Azoospermia	12.4 pg/ml	[36]
		Oligo-terato-asthenozoospermia + varicocele	12.9 pg/ml	[36]
		Oligo-terato-asthenozoospermia + genital infection	42.5 pg/ml	[36]
		Oligo-terato-asthenozoospermia + testicular lesion	18.2 pg/ml	[36]
IL-12				
	Healthy	***3.1 – 21.5 pg/ml***		
	Abnormal	***0 – 25 pg/ml***		
	Healthy		21.5 pg/ml 3.1 pg/ml 11 pg/ml	[42, 45, 55]
	Infertile	Ashthenospermia	11 pg/ml	[55]
		Oligospermic	6 pg/ml	[55]
		Oligoasthenospermia	0 pg/ml	[55]
		Azoospermic obstructive	25 pg/ml	[55]
		Azoospermic non-obstructive	18 pg/ml	[55]
IL-17				
	Healthy		*7.1 pg/ml*	[42]
IL-18				
	Healthy	***332.4 pg/ml***		
	Abnormal	***386.5 – 650.1 pg/ml***		
	Healthy		332.4 pg/ml	[64]

	Infertile	Genital infection	650.1 pg/ml	[64]
		Varicocele	389.6 pg/ml	[64]
		Cryptorchidism	390.1 pg/ml	[64]
		Mumps orchitis	386.5 pg/ml	[64]
		Idiopathic testicular lesion	377.7 mg/ml	[64]
		Klinefelter syndrome	417.5 pg/ml	[64]
IL-23				
	Healthy		5.87 pg/ml	[63]
	Infertile	Oligospermia	11.14 pg/ml	[63]
		50 – 55 y old males	497 IU/ml	
MIF				
	Healthy		3 µg/ml	[65]
	Infertile	Oligoterato-asthenoazoospermia + genital infection	7.67 µg/ml	[53]
TNF-α				
	Healthy	***ND – 37 pg/ml***		
	Abnormal	***0 – 62.5 pg/ml***		
	Healthy		ND ND 2.5 pg/ml 0 pg/ml 37 pg/ml 1.5 pg/ml 4.3 pg/ml 9 pg/ml 19.5 pg/ml	[8, 15, 45, 49, 54-56, 61]
		+ *Schistosoma haematobium* infection (low seminal egg secretion)	7.3 pg/ml	[54]
		+ *Schistosoma haematobium* infection (high seminal egg secretion)	78.3 pg/ml	[54]
	Infertile	Infertile	0 pg/ml 18.7 pg/ml	[56, 66]
		Normozoospermia	5.5 pg/ml 2.56 pg/ml	[57, 62]
		Normozoospermia (+ infection)	33.3 pg/ml	[61]
		Immunoinfertile (sperm abs)	0 pg/ml	[56]
		Infertile + genital infection	62.5 pg/ml 3.3 pg/ml	[49, 61]
		Ashthenospermia	0 pg/ml 5.88 pg/ml 3.43 pg/ml	[55, 57, 63]
		Oligozoospermic	0 pg/ml 6.27 pg/ml 5.6 pg/ml	[55, 62, 63]
		Oligoasthenospermia	0 pg/ml 1.69 pg/ml	[55, 57]
		Azoospermic obstructive	23 pg/ml	[55]
		Azoospermic non-obstructive	0 pg/ml	[55]

Table 2.1C

Cytokine/ Chemokine/ Factor	State of fertility/ disease	Concentration		Reference
IL-5				
	Healthy		*39.7 pg/ml*	*[42]*
IL-10				
	Healthy	***2.4 – 76.5 pg/ml***		
	Abnormal	***0.9 – 186 pg/ml***		
	Healthy		5.6 pg/ml 6.0 pg/ml 76.5 pg/ml 9.8 pg/ml 2.4 pg/ml 6.7 pg/ml 70.25 pg/ml	[35, 42, 45, 53, 55, 60]
		Fertile (+ infection)	5.2 pg/ml	[35]
		+ *Schistosoma haematobium* infection (low seminal egg secretion)	9.3 pg/ml	[54]
		+ *Schistosoma haematobium* infection (high seminal egg secretion)	26.8 pg/ml	[54]
	Infertile	Oligoterato-asthenoazoospermia	3.7 pg/ml	[53]
		Normospermia	20.58 pg/ml	[57]
		Azoospermic	8.2 pg/ml	[53]
		Azoospermic obstructive	186 pg/ml	[55]
		Azoospermic non-obstructive	93 pg/ml	[55]
		Genital infection + Oligoteratoasthenoazoospermia	0.9 pg/ml	[53]
		Leukospermia + infection	7.1 pg/ml	[35]
		Leukospermia	4.2 pg/ml 4 pg/ml	[35, 60]
		Ashthenospermia	158 pg/ml 10.80 pg/ml	[55, 57]
		Oligospermic	78 pg/ml	[55]
		Oligoasthenospermia	122 pg/ml 18.70 pg/ml	[55, 57]
TGF-β				
Total TGF-β1	***Healthy***	***61 – 238 ng/ml***		
	Abnormal	***112.7 – 130.7 ng/ml***		
Free TGF-β1	***Healthy***	***0.383 – 37 ng/ml***		
	Abnormal	***0.87 – 2.32 ng/ml***		
TGF-β1	Healthy	Total	219 ng/ml 92.4 ng/ml 238 ng/ml 85,548 pg/ml 71 ng/ml 61 ng/ml	[4, 58, 67-70]

		Free	0.383 ng/ml 1.82 ng/ml 2.3 ng/ml 2.0 ng/ml 9.22 ng/ml 37 ng/ml 1.6 ng/ml 0.9 ng/ml	[4, 8, 58, 60, 67-70]
	Infertile	Leukocytospermia	0.5 ng/ml	[60]
		Normozoospermia	0.554 ng/ml	[62]
		Oligozoospermia	0.534 ng/ml	[62]
		Secretory testicular pathology (total)	130.7 ng/ml	[68]
		Secretory testicular pathology (free)	2.32 ng/ml	[68]
		Epididymal occlusion (total)	105.8 ng/ml	[68]
		Epididymal occlusion (free)	0.87 ng/ml	[68]
		Non-obstructive genital tract pathology + testicular pathology (total)	112.7 ng/ml	[68]
		Non-obstructive genital tract pathology + testicular pathology (free)	1.69 ng/ml	[68]
TGF-β2	Healthy	Total	5.3 ng/ml 18 ng/ml 2.933 ng/ml 1.5 ng/ml	[4, 58, 67, 69, 70]
		Free	0.25 ng/ml 0.148 ng/ml 9 ng/ml 0.01 ng/ml 0.3 ng/ml	[4, 58, 67, 69, 70]
TGF-β3	Healthy	Total	172.2 ng/ml	[4, 58, 69]
		Free	3.5 ng/ml	[4]

Table 2.1D

Cytokine/ Chemokine/ Factor	State of fertility/ disease	Concentration		Reference
IL-8				
	Healthy	***7.2 – 2120 pg/ml***		
	Diseased	***28 – 9490 pg/ml***		
	Healthy		2120 pg/ml 720 pg/ml 1300 pg/ml 664.59 pg/ml 4452 pg/ml 7.2 pg/ml 900 pg/ml 1583 pg/ml 1900 pg/ml 1150 pg/ml 491 pg/ml	[8, 15, 18, 36, 37, 42, 45, 55, 61, 63, 67]
	Infertile	Infertile (no pathogen infection)	28 pg/ml	[37]
		Infertile + pathogen infection	34 pg/ml, 803 pg/ml	[37, 61]
		Normozoospermia	2005 pg/ml 6935 pg/ml	[15, 57]
		Normozoospermia (+ infection)	656 pg/ml	[61]
		Leukospermia	1650 pg/ml	[18]
		Non-leukospermia	610 pg/ml	[18]
		Ashthenospermia	2850 pg/ml 919.2 pg/ml	[55, 57]
		Oligozoospermic	1450 pg/ml 1113.86 pg/ml 2058 pg/ml	[15, 55, 63]
		Oligoasthenospermia	1750 pg/ml 1049.7 pg/ml	[55, 57]
		Azoospermic obstructive	2050 pg/ml	[55]
		Azoospermic non-obstructive	2100 pg/ml	[55]
		Azoospermia	4950 pg/ml	[36]
		Oligo-terato-asthenozoospermia + varicocele	5450 pg/ml	[36]
		Oligo-terato-asthenozoospermia + genital infection	9450 pg/ml	[36]
		Oligo-terato-asthenozoospermia + testicular lesion	5450 pg/ml	[36]
IP-10				
	Healthy		1.8 nM	[71]
I-TAC				
	Healthy		0.6 nM	[71]
MCAF/MCP-1				
	Healthy		2.78 ug/L	[8, 18, 42]

			8000 pg/ml 3251 pg/ml	
	Infertile	Leukospermia	11.19 ug/L	[18]
		Non-leukospermia	3.24 ug/L	[18]
MIG				
	Healthy		25 nM	[71]
MIP-1α				
	Healthy		375 pg/ml 6.9 pg/ml	[8, 42]
MIP-1β				
	Healthy		400 pg/ml 54.1 pg/ml	[8, 42]
RANTES				
	Healthy		400 pg/ml 119.9 pg/ml	[8, 42]
SDF-1α				
	Healthy		5087.7 pg/ml	[42]

Table 2.1E

Cytokine/ Chemokine/ Factor	State of fertility/ disease	Concentration		Reference
BDNF				
	Healthy		3.4 ng/ml	[72]
	Infertile	Oligoasthenozoospermia	2.8 ng/ml	[72]
		Asthenzoospermia	3.4 ng/ml	[72]
EGF				
	Healthy		34.9 ng/ml	[73]
	Infertile		26.5 ng/ml	[73]
		Infertile + abs	37.4 ng/ml	[73]
G-CSF				
	Healthy		250 pg/ml 47.5 pg/ml	[42, 58]
	Infertile	Normoszoospermia	82 pg/ml	[62]
		Oligozoospermia	50 pg/ml	[62]
GM-CSF				
	Healthy		5.6 pg/ml 200 pg/ml 1.5 pg/ml	[8, 42, 58]
IL-2				
	Healthy	***ND – 1.2 pg/ml; ND – 261 fmol/ml***		
	Abnormal	***0.89 – 415.1 pg/ml; 219 – 444.3 fmol/ml***		
	Healthy		ND 251.3 fmol/ml 261 fmol/ml 215.2 pg/ml 6.2 pg/ml 3.6 pg/ml 1.2 pg/ml	[10, 36, 37, 42, 54, 59, 74]
	Infertile	Infertile	444.3 fmol/ml 241 fmol/ml 17 pg/ml	[37, 59, 74]
		Infertile + pathogen infection	26 pg/ml	[37]
		Normospermia	0.76 pg/ml	[57]
		Oligoasthenospermia	0.72 pg/ml	[57]
		Asthenospermia	0.89 pg/ml	[57]
		Azoospermia	219 fmol/ml 390 pg/ml	[36, 59]
		Oligo-terato-asthenozoospermia + varicocele	415.1 pg/ml	[36]
		Oligo-terato-asthenozoospermia + genital infection	405 pg/ml	[36]
		Oligo-terato-asthenozoospermia + testicular lesion	389.1 pg/ml	[36]
sIL-2R				

	Healthy	***25.9 – 425 units/ml***		
	Infertile	***22.1 – 310 units/ml***		
	Healthy		425 units/ml 5.5 fmol/ml 350 units/ml, 25.9 units/ml	[44, 53, 59, 67]
	Infertile	Infertile	5.6 fmol/ml, 22.1 units/ml	[44, 59]
		Oligoterato-asthenoazoospermia	310 units/ml	[53]
		Genital infection + Oligoterato-asthenoazoospermia	240 units/ml	[53]
		Azoospermic	310 units/ml	[53]
sIL-6R				
	Healthy	***4 – 2250 pg/ml***		
	Diseased	***4.8 – 2250 pg/ml***		
	Healthy		2250 pg/ml 4 pg/ml	[53, 59]
	Infertile	Infertile	4.8 pg/ml	[59]
		Oligoterato-asthenoazoospermia	1900 pg/ml	[53]
		Genital infection + Oligoterato-asthenoazoospermia	2100 pg/ml	[53]
		azoospermic	2250 pg/ml	[53]
IL-7				
	Healthy	2365.8 pg/ml		[42]
IL-11				
	Healthy	***18.5 – 6472 pg/ml***		
	Abnormal	***21.1 – 9529 pg/ml***		
	Healthy		6472 pg/ml 18.5 pg/ml 3575 pg/ml	[36, 45, 55]
	Infertile	Ashthenospermia	4602.5 pg/ml	[55]
		Oligospermic	5815 pg/ml	[55]
		Oligoasthenospermia	4125 pg/ml	[55]
		Azoospermic obstructive	9529 pg/ml	[55]
		Azoospermic non-obstructive	4991.5 pg/ml	[55]
		Azoospemia	24.9 pg/ml	[36]
		Oligo-terato-asthenozoospermia + varicocele	21.1 pg/ml	[36]
		Oligo-terato-asthenozoospermia + genital infection	215.6 pg/ml	[36]
		Oligo-terato-asthenozoospermia + testicular lesion	22.7 pg/ml	[36]
IL-13				
	Healthy		*3.2 pg/ml*	*[42]*
NGF				
	Healthy		0.82 ng/ml	[46]
	Infertile	Oligoasthenozoospermia	0.68 ng/ml	[46]

		Asthenozoospermia	0.79 ng/ml	[46]
VEGF				
	Healthy		772 ng/ml	[58]
	Infertile	Normozoospermia	523.94 ng/ml	[62]
		Oligozoospermia	474.64 ng/ml	[62]

2.4 Factors in semen modulate immunity essential to establishing fertilization

Factors in seminal fluid must be present at optimal concentrations in order to achieve successful reproduction. Fertility is more than just a function of sperm count; the components of seminal fluid not only control the concentration of sperm in semen, but also influence the quality of the spermatozoa and alter the environment within the FRT to orchestrate tolerance of the paternal antigens in support of fertilization and embryo implantation. Many studies of healthy human males, infertile human males, and animals have provided insights into the intricate processes that take place in the FRT during reproduction.

Events begin with the deposition of semen in the FRT. Initially, semen is recognized by the local female innate immune system as a foreign entity due to the presence of paternal antigens and MHC class I and II on spermatozoa [75]. As a result, an inflammatory reaction is incited, characterized by a massive infiltration of neutrophils that are likely recruited by the high concentrations of IL-8 (Table 2.1D) and GM-CSF (Table 2.1E) found in seminal fluid [76]. The initial phase of inflammation is important to clear excess and abnormal sperm as well as various waste products found within semen [70, 76]. It is vital, however, that the inflammatory phase be quickly resolved, as uncontrolled inflammation could lead to the clearance of the healthy sperm cells by neutrophils and result in a reduced capacity for fertilization. In order to suppress the female immune system and allow the paternal antigens to go unrecognized, a state of tolerance must be induced, likely mediated by the pleiotropic cytokine TGF-β. Both pro-TGF-β and active TGF-

β are secreted into semen by the seminal vesicles and prostate, and are present at very high concentrations within the seminal fluid (Table 2.1C) [76, 77]. In combination with seminal IL-15, TGF-β tilts the immune balance from the initial Th1 response to a Th2 type response through the induction of regulatory T (T_{reg}) cell differentiation of T cells from the periphery [5, 77]. As T_{reg} cells increase in number, a concurrent opposing shift in the local immune cell population results in the suppression of Th17 switching, the overabundance of which has been correlated with preeclampsia [78]. Additional studies suggest that semen may further bias a type 2 response by inducing the production of type 2 cytokines from local and intraepithelial T cells and dendritic cells (DCs), possibly through semen-associated prostaglandins [58, 79]. These studies highlight the active role of semen in inducing the immune changes that prevent the semi-allograft fetus from being recognized as a foreign antigen and protect it from clearance [76].

TGF-β, however, is in no way the only important immunomodulatory factor found in semen. Another important suppressive component found in high concentrations is prostaglandin E_2 (PGE_2), which also originates in the seminal vesicles (Table 2.1A). Prostaglandins are key in dampening the immune response through inhibition of macrophage cytokine production and T cell proliferation [70, 76, 77, 80, 81]. Like IL-15, PGE_2 also works in combination with TGF-β to promote tolerance of paternal and fetal antigens, contributing to the differentiation and expansion of T_{reg} cells in the FRT [5, 77]. Abnormalities in any one of the functions directed by the factors in seminal fluid, such as embryo implantation and development, could lead to a state of dysregulation, as evidenced by the many

types of male infertility that have been identified and described in literature (Table 2.1).

2.5 Changes in semen-associated immune factors correlated with infertility in the MRT

Due to the complex composition of seminal fluid, male infertility may be correlated with an increase or decrease in a multitude of factors noted for their immunomodulatory activities (Table 2.1). The presence of interleukin 1β (IL-1β), a pro-inflammatory cytokine, has been inversely correlated with sperm motility (Table 2.1B). Similarly, increases in interleukin 6 (IL-6), possibly secreted by lymphocytes in semen or hormonally regulated Sertoli cells in the MRT, have been correlated with decreases in sperm count and motility, as well as decreases in semen volume (Table 2.1B) [35, 55, 56]. Interleukin 8 (IL-8), a chemotactic cytokine found at higher levels in the semen of individuals diagnosed with infertility due to leukocytospermia, has been suggested to be responsible for recruiting excessive numbers of white blood cells (WBCs), resulting in abnormally high T-lymphocyte counts in semen as well as a decrease in spermatozoa viability (Table 2.1D) [56]. Increased IL-8 has also been correlated with increases in pro-inflammatory cytokines interleukin 1α (IL-1α) (Table 2.1B) and granulocyte colony-stimulating factor (G-CSF) (Table 2.1E), which may be released into semen by macrophages or endothelial cells lining the MRT. IL-1α and G-CSF are responsible for activating T cell proliferation and stimulating neutrophil production, which are both key to eliciting an inflammatory immune response [82]. Increased interleukin

18 (IL-18), which was shown to be present at higher concentrations in semen of men suffering from combined infertility/urogenital infections, was correlated with decreased sperm concentration and mobility (Table 2.1B) [64]. Several studies have noted an increase in tumor necrosis factor alpha (TNF-α) in semen in conjunction with low or undetectable sperm counts (Table 2.1B). TNF-α has also been correlated with reductions in testosterone and sperm motility, alterations in sperm morphology, and increases in reactive oxygen species (ROS) [35, 55, 63]. Similarly, increases in interferon gamma (IFN-γ) across multiple infertility conditions were also associated with decreased sperm count, motility, and morphology (Table 1B) [55].

The protein monocyte chemotactic and activating factor (MCAF) provides a particularly illustrative example of a biological factor contained in semen that may impact reproductive fitness through variations in concentration. Levels of MCAF in seminal fluid of men with leukocytospermia are increased over those of other infertile-type conditions or fertile males (Table 1D), indicating that specific infertility diagnoses can be associated with specific cytokine concentrations [18]. Increased levels of MCAF may originate from monocytes, endothelial cells, or fibroblasts present in the MRT and be involved in protecting the reproductive tract from infection by bacterial pathogens [18]. The role of MCAF in reproductive biology, however, is not limited to the male. It has been speculated that once semen has been deposited into the FRT, MCAF may be responsible for the local recruitment and activation of monocytes, which are important in clearing excess sperm [18]. In leukocytospermia, MCAF stimulation has also been associated with the release of

IL-6 from monocytes, which in conjunction with increased IL-8, results in further activation and differentiation of monocytes into macrophages and recruitment of neutrophils [18]. An increase in the local presence and activation of phagocytes could result in increased clearance of sperm cells, hindering fertility. Cumulatively, these factors and effects directly influence mechanisms that may alter local immune responses that affect not only reproductive events, but may also contribute to the risk of STD pathogen infection within the FRT [29].

2.6 Effects of seminal factors in the FRT

In addition to its direct influence on immune cells, semen can also have indirect effects on the immune response mediated through the epithelial cells lining the reproductive tract of the female. While the FRT is a large and complex environment, the epithelial lining can essentially be broken down into three main regions: the vagina, which has the largest surface area in the FRT and is characterized by a squamous epithelium that can be up to 25 cell layers thick; the ectocervix, which encompasses the portion of the epithelium that transitions from a multi-layer squamous phenotype into a simple columnar phenotype characterized by low tight junction expression; and the endocervix, which is described as a single columnar epithelial layer with high levels of tight junction expression [83]. Interestingly, our group and others have observed that each of these regions is not only unique in phenotype or tight junction expression, but in responsiveness to stimuli, such as seminal fluid, revealing that the epithelium is more than just a mechanical barrier – it is also a dynamic tissue [84]. In fact, seminal fluid seems to elicit this response on tight junctions on epithelial cells in a

variety of environments [85]. When epithelial cells derived from the three main regions of the reproductive tract were incubated with seminal fluid in submerged culture, a wide array of factors were released, the most predominant of which were the inflammatory cytokines [58, 84]. However, the concentrations and specific cytokine release profiles were unique to each region [84]. In one study, cells derived from the ectocervix were the most responsive compared to cells of the vaginal tract. These results suggest that the vaginal epithelium, which is regularly exposed to bacteria, pathogens, irritants, and foreign entities (including semen), has a greater tolerance for damage or inflammatory stimuli [84]. The cervix, on the other hand, is considered to be essentially sterile due to its remote placement relative to the vaginal opening, and is protected from external insults by a layer of mucus. Because this region is exposed to a lower level of stimuli relative the vaginal epithelium, the tolerance of the epithelium might be lower and its responsiveness to insults correspondingly higher [84]. Studies have shown that even in the cervix there are regional differences, with the endocervix (higher up in the FRT) having a greater response to foreign stimuli [86]. Perhaps this robust response is due to the fact that the upper cervical region sees fewer foreign stimuli than the lower region and stands poised to provide a more effective response to stop colonization of the upper cervix [86].

Factors that are released by epithelial cells in a basolateral direction in response to apical exposure to semen also orchestrate reproductive inflammatory/tolerance events by influencing local immune cells [87]. For example, the transition of uterine natural killer cells (uNK) from a cytotoxic to a “helper”

phenotype is essential to fetal development [88], insofar as having uNKs recognize fetal development as “self,” or non-foreign, and therefore not attacking the developing fetus through induced tolerance. During embryogenesis, uNK cells release IFN-γ to aid in angiogenesis and vascularization of the uterus in order to support the developing fetus [88, 89]. Although it remains controversial, the shift in uNK phenotype leading to tolerance may be induced by IL-15 and IL-7 [90]. Release of IL-15 from the epithelium is increased 22-fold in the subepithelial space as early 4 h post-semen exposure [91]. As mentioned previously, the combination of IL-15 and TGF-β increases T cell switching to a T_{reg} phenotype, further contributing to key events that initiate fetal tolerance [77]. Interestingly, the number of T_{regs} and uNK cells present in the uterus are higher relative to the peripheral blood, implicating the intensive role these cell types play in local reproductive immunity [92]. The shift from inflammation to tolerance in the FRT during semen exposure has been likened to the changes that occur in the tumor microenvironment, both of which provide protection from immune clearance and allow cells to proliferate in a similar manner [93]. Combined, the activity of the reproductive epithelium added to the effects of factors contained in semen creates a biphasic local immune response that is key to clearing excess sperm and essential to developing tolerance to the nascent fetus in the FRT during reproduction.

In the context of pathogen transmission, the same factors that are important to reproduction may also contribute to susceptibility to sexually transmitted infections (STIs). When CD4+ T cells were incubated directly in seminal plasma,

there was an observed increase in CCR5 surface expression, resulting in preferential R5 infection in the presence of human immunodeficiency virus type 1 (HIV-1) [7]. There is also evidence that the same type 2 responses that are responsible for tolerance of sperm may also protect the virus from clearance. Prostaglandins in semen that drive a local type 2 response, as described previously, may also inhibit IL-12 release and upregulate IL-10 production by DCs, possibly reducing the capacity for clearance of virus [79]. Combined with the data demonstrating that IL-7 – which is increased in the seminal fluid of HIV-1-infected males and also released by the epithelium after semen exposure – can enhance HIV-1 transmission to T cells in cervicovaginal explants [94], these findings further highlight the potential effects of combined factors from both seminal fluid and the reproductive epithelium in directly modulating transmission of HIV-1, as well as other STD pathogens.

2.7 Future directions

Studies that strive for a better understanding of the interplay between seminal factors and the FRT will provide new insights into reproductive biology as well as failures in reproduction attributable to disease or infection. Such studies will also give rise to the development of new model systems in which critical aspects of this important interplay can be readily manipulated and studied. For example, in vitro studies using a simple model consisting of semen and cervicovaginal epithelial cells may attribute a failure to modulate key innate immune responses in a timely manner to decreased concentrations of TGF-β in

seminal plasma. Such information could be the catalyst for the development of new drugs that effectively treat infertility. This review highlights the importance of the major constituents of semen and provides the first stepping stones toward future experiments of this nature.

Outside of reproduction, immunomodulatory factors in semen may also affect key events that influence the transmission of STD pathogens, such as HIV-1, resulting in an increased or decreased risk of male-to-female transmission. Having greater knowledge about the dynamics of biologically active factors in semen will increase our understanding of not only reproductive biology, but also STD pathogen transmission. More detailed information about the role of semen in modulating the risk of pathogen transmission will likely reveal new strategies for preventing STD acquisition by women. For example, a greater understanding of seminal factors that result in FRT inflammation and the chemotaxis of HIV-1-susceptible immune cells to the cervicovaginal epithelium may provide a path toward the development of next generation topical microbicides that reduce semen-associated immune cell recruitment and reduce the risk of male-to-female HIV-1 transmission. It is our hope that this review will also serve as a supporting resource for these types of studies.

2.8 Summary

Semen is a biologically active fluid capable of orchestrating and influencing immune responses within the FRT. Some of the numerous agents in semen that profoundly affect the immunological environment of the FRT include cytokines,

chemokines, growth factors, and other biologically active molecules. Said components disrupt the normal immune response of the female, which may leave her susceptible to further complications. Although the MRT is highly compartmentalized, semen composition can be influenced by reproductive tract disease and conditions outside the MRT, resulting in changes in concentration of key seminal components that are important in guiding reproductive processes in the FRT. Significant changes in factors present in semen may result in infertility or reproductive dysfunction. Similarly, indirect changes induced by semen, such as the release of factors by the epithelium or changes in immune cell populations, may also influence the delicate pro-inflammatory and anti-inflammatory phases of reproduction.

Chapter 3

Roles for semen in altering immune cell populations found in the female reproductive tract

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3.1 Abstract

When semen comes in contact with the female reproductive tract (FRT), it activates a biphasic immune response. Immune cell recruitment to the site of deposition is followed by a dampening of the immune response. In this regard, semen alters the immune cell populations already present in the female reproductive tract. The already high levels of $CD8^+$ and $CD4^+$ T lymphocytes are increased in numbers, along with natural killer cells, macrophages, and other antigen presenting cells. This is especially true in the immune microenvironment of the transformation zone, where the squamous epithelium of the lower cervix becomes a single columnar epithelium in the upper cervix. When exposed to seminal factors, the transformation zone becomes a “hot spot” for human immunodeficiency virus type 1 (HIV-1) transmission. In this review, we cover the transduced effect semen has on cells in the subepithelial space of the FRT and its potential for modulating pathogen transmission, with a focus on HIV-1. This review also briefly examines changes in seminal content due to HIV-1 infection and how the changes in semen content may further alter the risk of HIV-1 transmission from male-to-female. By understanding semen-dependent changes in the subepithelial environment of the FRT, we can draw conclusions about early events occurring in male-to-female pathogen transmission and, perhaps, identify mechanisms as possible targets for prevention therapies.

3.2 Introduction

Semen is a dynamic fluid comprised of multiple cytokines, chemokines, growth factors, and whole cells. One function of semen is to serve as a vehicle for sperm delivery to a recipient female, who will, in turn, utilize the provided paternal genetic material to generate an embryo and initiate the process of reproduction [29]. When applied to the female reproductive tract (FRT), semen initiates tissue remodeling and release of cell signaling factors that are part of the innate immune response [95].

The innate immune response in the FRT is activated by interactions between the genital epithelium and foreign stimuli. To that end, the FRT epithelium is lined with polarized cells that express pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) [96]. These sensors allow the female to quickly react to different pathogens introduced into the FRT. A response to foreign stimuli may include activation of transcription factors and the production of cytokines and chemokines [29, 96, 97]. The cascade of cytokines produced will be reliant on the type of pathogen encountered and the specific pattern of PRR activation. While semen is not a pathogen, it is a source of “non-self" antigens and can be considered analogous to a pathogen with respect to innate immune responses and inflammation in the FRT. Upon introduction of semen into the FRT, an inflammatory response is initiated to clear the semen [98, 99] (Fig. 3.1A). However, to facilitate successful reproduction, semen must counter the innate immune response by “re-writing” the female immune response to accept the paternal antigens as “self” (Fig. 3.1B).

To counteract the inflammatory response, semen initiates a biphasic immune response, meaning that inflammation is soon abated, allowing the female to tolerate the presence of sperm [28, 100]. However, these changes in the FRT may make for an environment more suitable for pathogen transmission. In fact, by inducing tolerance in the latter half of the biphasic immune response, semen inadvertently creates an environment suitable for pathogen transmission. In the case of human immunodeficiency virus type 1 (HIV-1) induction of tolerance will produce an influx of T lymphocytes to the site of deposition [5, 28], which will provide more target cells for HIV-1 to infect; further facilitating disease transmission. Therefore, semen-dependent changes in the FRT, that are part of the normal process of fertilization, lead to changes in the risk of pathogen transmission [87].

Previous studies of semen and its constituents in conjunction with HIV-1 transmission have mainly focused on how HIV-1 changes the composition of semen [1, 42, 101]. Rarely have studies been used to determine how these changes affect the FRT or their downstream effects. Furthermore, almost no connections have been made between changes in semen content and changes in the subepithelial immune cell populations in the FRT, and how these changes may increase the risk of male-to-female HIV-1 transmission.

Figure 3.1

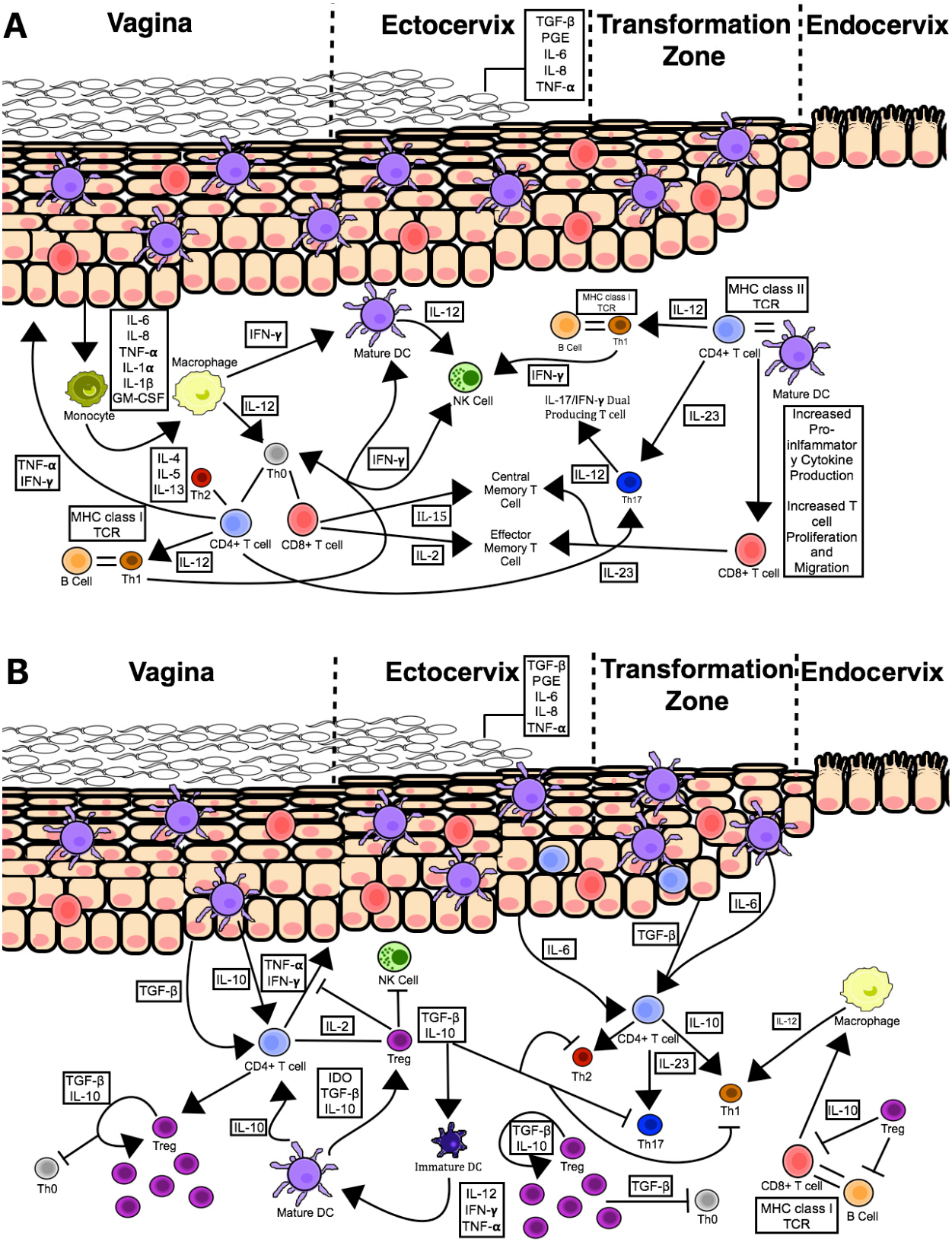
A
Vagina
Ectocervix
Transformation Zone
Endocervix
TGF-β
PGE
IL-6
IL-8
TNF-α
IL-6
IL-8
TNF-α
IL-1α
IL-1β
GM-CSF
Monocyte
Macrophage
IFN-γ
IL-12
Mature DC
NK Cell
MHC class I TCR
B Cell
Th1
IL-12
MHC class II TCR
CD4+ T cell
Mature DC
IFN-γ
IL-17/IFN-γ Dual Producing T cell
IL-23
Increased Pro-inlfammatory Cytokine Production
Increased T cell Proliferation and Migration
TNF-α
IFN-γ
IL-4
IL-5
IL-13
Th2
Th0
IFN-γ
Central Memory T Cell
IL-12
Th17
MHC class I TCR
CD4+ T cell
CD8+ T cell
IL-15
Effector Memory T Cell
IL-2
B Cell
Th1
IL-12
IL-23
CD8+ T cell
B
Vagina
Ectocervix
Transformation Zone
Endocervix
TGF-β
PGE
IL-6
IL-8
TNF-α
IL-6
TGF-β
IL-6
TGF-β
IL-10
TNF-α
IFN-γ
NK Cell
IL-2
TGF-β
IL-10
CD4+ T cell
Treg
CD4+ T cell
IL-10
IL-12
Macrophage
Th2
IL-23
TGF-β
IL-10
IDO
TGF-β
IL-10
Th1
IL-10
Treg
Th0
Treg
IL-10
Immature DC
TGF-β
IL-10
Th17
Treg
Mature DC
IL-12
IFN-γ
TNF-α
TGF-β
Th0
CD8+ T cell
MHC class I TCR
B Cell

Fig. 3.1. Flow diagram of semen's biphasic effect in the female reproductive tract

In the (A) inflammatory response factors in semen elicit a pro-inflammatory cytokine cascade from the epithelium that interact with monocytes, DCs, T lymphocytes, and macrophages. The resulting effect is a network of interplay among all immune cells present causing an increased release of TNF-α and interferon gamma (IFN-γ), promoting a further inflammatory response. Shortly after inflammation and the recruitment of T lymphocytes, seminal TGF-β and IL-15 produce an anti-inflammatory response from the epithelium causing a (B) tolerogenic environment. T cells become Tregs, which through the production of IL-10 and TGF-β, dampen the immune response by depleting the subepithelium of all other T lymphocyte lineages while simultaneously auto-expanding the Treg pool.

3.3 Immune Cell Changes in the Female Reproductive Tract

3.3.1 Immune cells present in the female reproductive tract before exposure to semen

Examining the immune cells present in the subepithelium of the FRT before and after semen deposition will allow one to draw conclusions about the effect semen has on the changes in the local immune environment in the subepithelial tissue. Prior studies of the subepithelium revealed that T lymphocytes, natural killer cells (NKs), macrophages, B lymphocytes, granulocytes, and dendritic cells (DCs) make up the majority of the populations present in the FRT [102]. However, the FRT can be divided into three anatomically distinct regions: two being the vagina and cervix, with the cervix being further subdivided into the ecto- and endocervix. Furthermore, in the cervix there is a transformation zone: the point where the squamous epithelium of the ectocervix (lower portion of the cervix) becomes the single columnar epithelium of the endocervix (higher portion of the cervix).

The different regions of the FRT are not just anatomically distinct; they each represent immune microenvironments. Each region - the vagina, ectocervix, endocervix, and transformation zone - respond differently to stimuli and, as such, have varied populations of immune cells that make up their immune microenvironments. Pertaining to $CD3^+$ lymphocytes, unlike in peripheral blood, the entirety of the FRT has more $CD8^+$ T lymphocytes than $CD4^+$ T lymphocytes [102]. In the vagina, increased numbers of $CD8^+$ T lymphocytes are found in the lamina propria, and, although some $CD4^+$ T lymphocytes can also be found in the lamina propria, the $CD8^+$ T lymphocytes outnumber the $CD4^+$ cells [102]. $CD68^+$

macrophages and HLA-DR$^+$ DC-like cells can also be found in small numbers in the vagina [102]. Toward the upper portion of the vagina, close to the ectocervix, the immune cell populations shift slightly. Increasing in numbers in the lamina propria are CD1a$^+$ immature DCs, yet still, CD8$^+$ cells make up the majority of lymphocytes found in the lamina propria and epithelium [102]. Of these T lymphocytes, the majority are memory T cells (CD45RO$^+$). However, naïve T lymphocytes (CD45RA$^+$) are still present [102], albeit in smaller numbers.

As the epithelium transitions from the vagina to the cervix, the immune microenvironment shifts. Some similarities between the vagina and ectocervix can be seen, i.e. CD3$^+$ T lymphocytes are most abundant, with 40% of the population being CD4$^+$ T lymphocytes and 60% being CD8$^+$ T lymphocytes in the ectocervix [102]. The majority of the CD4$^+$ T lymphocytes are of the effector/memory or effector/T cell phenotype [103]. Of the other CD45$^+$ mononuclear cells, only ~2.7% are NK cells and ~0.9% are CD19$^+$ B lymphocytes [102]. However, the ectocervix has abundantly more CD4$^+$ T lymphocytes and CD1a$^+$ DCs as compared to the vagina [102].

The biggest differences in the FRT, with respect to immune microenvironments are found further up the FRT in the endocervix. The endocervix prevents infections from pathogens ascending the FRT by utilizing mucus glands that trap foreign stimuli [103]. Therefore, the endocervix doesn't usually encounter pathogens, unlike the ectocervix, which is routinely exposed to probiotics, commensal flora, and pathogens [103]. Perhaps as a result of the sterility of the endocervix, there are half as many CD3$^+$ T lymphocytes found in the upper cervix

relative to the lower cervix [102]. Therefore, $CD4^+$ and $CD8^+$ T lymphocytes are more abundant in the ectocervix than in the endocervix [103]. B lymphocytes follow the same pattern. However, NK subsets were about even in numbers between the ecto- and endocervix [103].

The squamous epithelia of the vagina and ectocervix also harbor interstitially dispersed cells among the layers of cells. For example, in the vagina and ectocervix, Langerhans cells can be found in abundance in between adjacent cells, whereas in the endocervix Langerhans cells may be present but in fewer numbers [102]. The interstitial location of the Langerhans cells may permit them to reach the luminal side of the FRT, where they can interact with foreign stimuli and act as first responders [104]. One such interaction may be between Langerhans cells and HIV-1. Langerhans cells are thought to be initial targets for HIV-1, perhaps because they sample the luminal side of the FRT and are thus the first immune cells HIV-1 may contact in the FRT [105, 106] . It is unclear, however, if Langerhans cells are the first cells to become infected by HIV-1. It is therefore unknown if Langerhans cells may facilitate transmission of the disease. It is known, however, that Langerhans cells may act as a reservoir for HIV-1 [105, 107] . In doing so Langerhans cells act as a primary defense against HIV-1 and prevent the virus from reaching target $CD4^+$ T lymphocyte populations in the subepithelium [102, 105, 107] .

Again, other types of immune cells present in the FRT are found at varying concentrations in each tissue and region. Immune cell populations in the vagina and cervix generally have T lymphocytes in the greatest abundance, followed by

NKs, with B lymphocytes as the least concentrated in number [102]. In the context of HIV-1, T lymphocytes are important populations to focus on because $CD4^+$ T lymphocytes (T helper cells) are primary targets for the virus since they express CD4, which is one of the cell surface co-receptors necessary for HIV-1 entry and infection [108]. In fact, recent ex vivo studies have shown that infection of immune cells by HIV-1 occurred more frequently in T cell subsets than in other types, suggesting that T helper cells may be the first to become infected by HIV-1 [109, 110]. In that study, $CD4^+$ T_h17 cells were the most readily infected subset [109, 110]. $CD4^+$ T_h17 cells are involved in the innate immune response of the FRT and vary in concentration across the FRT, with the greatest concentration being harbored by the ectocervix [109, 110]. Because these cells are involved in maintaining mucosal surfaces as well as clearance of pathogens at said surfaces [109, 110], their abundance in the ectocervix is consistent with their function.

In fact, $CD4^+$ T_h17 cells are one of the T lymphocyte subsets found in great numbers in the transformation zone [103], where the ectocervix becomes the endocervix. The immunological microenvironment of the transition zone, unlike the surrounding tissues, has an increased concentration of T lymphocyte populations and antigen presenting cells (APCs) [103]. Interstitially placed in the transition zone are an abundance of $CD8^+$ T lymphocytes [102, 103], which, again, make up the most abundant immune cells in the FRT. With increased numbers of all cell types in the transformation zone, it seems plausible to identify this region as the central hub for cell-mediated immunity in the FRT [103]. Consistent with this concept, inflammation associated with cervicitis and vaginitis was found to result in

increased intraepithelial numbers of $CD8^+$ and $CD4^+$ T lymphocytes and APCs in the transformation zone [107]. These pathogen-induced responses suggest that the transformation zone may also be a “hot-spot” [111] for HIV-1 transmission during inflammation, as well as the central hub for cell mediated immunity [103]. These findings are backed up by non-human primate studies that showed that simian immunodeficiency virus (SIV) transmission, which occurs within hours of the viral challenge, takes place predominantly in across the ectocervical epithelium [112, 113]. In related reproductive biology studies in mice, an influx in T lymphocytes was seen in the ectocervix within four hours after coitus [5, 77, 114]. Cumulatively, these results point to the transformation zone as a potential hot-spot for HIV-1 infection.

3.3.2 Seminal factors interact with the FRT to induce a pro-inflammatory environment in the subepithelium

Therefore, semen influences immune cell populations in the subepithelium of the FRT by acting through the mucosal epithelial tissues. Biologically active factors in semen are unlikely to penetrate the epithelium to influence underlying immune cell populations [29]. In fact, the generation of Treg cells in the ectocervix so quickly after coitus in animal models is direct evidence of semen’s effect on female immunity [5, 77] .

Semen is comprised of a multitude of biologically active factors: hormones, cytokines/chemokines, fructose, vitamin D, and immune cells [29]. Together, these factors induce a pro-inflammatory environment in the subepithelium of the FRT [87, 99, 114] (Fig. 3.1A). However, variability in semen may also account for

differences in immune activation [49, 82, 115]. Changes in fertility, age, sexually transmitted diseases (STDs), medication, and diet [29] are only a few variables that induce variability in semen. It is known that infertility in males may be attributed to adverse changes in cytokine and chemokine content in semen [29, 63]. By extension, innate immune responses mounted in the FRT to these individuals may differ from the immune responses mounted against normal, healthy male semen. As result of altered semen content, the innate immune response in the FRT becomes altered. This may result in an over-abundance of immune cell recruitment to the subepithelium of the FRT. In the context of HIV-1 transmission, more target $CD4^+$ T lymphocytes may become available due to the higher pro-inflammatory nature of semen inducing an inflammation response through the epithelial barrier.

In mice, colony stimulating factor 2 (CSF2), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-1 alpha (IL-1α) were all detected by quantitative RT-PCR in cervical tissues following coitus [28]. This led to the recruitment of macrophages, DCs, and memory T lymphocytes to the cervix [28]. Normal, healthy semen is able to induce changes in the FRT that would further facilitate HIV-1 transmission via induction of $CD4^+$ T lymphocytes. Thus also goes back to the latter stage of the biphasic effect semen elicits, which is to dampen inflammation to allow fertilization to occur (Fig. 3.1B).

The biphasic effect of semen starts with inducing inflammation through seminal constituents (Fig. 3.1A). Reproductive studies in mice show that semen induces a pro-inflammatory response from the FRT that is absent in virgin mice [99]. In fact, semen from mice without seminal vesicles failed to induce

macrophages and DCs after mating, suggesting that the seminal vesicle is responsible for providing the constituents in semen that elicit an inflammatory response in the FRT [99]. Furthermore, granulocyte-macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNF-α) are also not generated from the female epithelium when seminal vesicle deficient male semen interacts with the FRT [99]. Cumulatively, semen expands the immune cell populations through interactions with the FRT epithelial barrier. During the initial inflammation stage of semen exposure, pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), are released from the epithelium (Fig. 3.1A). These cytokines interact with immune cells in the subepithelium, which then produce their own cytokines (Fig. 3.1A). These cytokines then go on to interact with DCs, NK cells, naïve T lymphocytes (T_h0), and others (Fig. 3.1A). The inflammatory response recruits T lymphocytes to the site of semen deposition and expands the pool of T_h0 cells into T_h1, T_h2, and T_h17 subsets (Fig. 3.1A) to further enhance inflammation.

However, seminal interleukin-15 (IL-15) and transforming growth factor beta (TGF-β) acting through the epithelium will also induce a tolerogenic environment (Fig. 3.1B). Interestingly, increased epithelial production of IL-15 (~22-fold) in a basolateral direction in response to apical exposure to semen has also been reported [23]. In fact, early recruitment of $CD3^+$ T lymphocytes to the site of semen deposition will further facilitate a tolerogenic state through the induction of T regulatory (Treg) cells [5, 77] (Fig. 3.1B). The pool of T helper and Treg cells expands with the basolaterally produced IL-15, interleukin 10 (IL-10), and TGF-β,

as a response to semen. The expanded pool of Tregs further act to shut down inflammatory inducing immune cells such as T_h1 and T_h2 cells (Fig. 3.1B). In fact, Tregs dampen the inflammatory cascade through inhibition of most cells types and processes (Fig. 3.1B), thereby leaving the environment in a state to tolerate the semen, until full clearance. Therefore, healthy male semen already primes the FRT for immune tolerance and may further facilitate HIV-1 transmission from male-to-female. The increased amount of HIV-1 target cells abundant in the subepithelium coupled with a severe reduction in female defenses allows HIV-1 to enter and replicate in T cells.

3.3.3 Modulation of seminal factors by HIV-1 may have downstream effects in the FRT

Studies have also categorized differences in seminal constituents between healthy males and those with HIV-1. Specific factors like IL-1α [8, 10, 29], interleukin-4 (IL-4) [6, 29], IL-6 [6, 10, 14, 15, 24, 29], interleukin-7 (IL-7) [6, 24], interleukin-8 (IL-8) [6, 10, 14, 15, 24, 29], interleukin-16 (IL-16) [24], macrophage inflammatory protein alpha and beta (MIP-1α and MIP-1β) [8, 24], regulation on activation normal T cell expressed and secreted (RANTES) [8, 13, 24], and TGF-β [8, 24] are all increased in HIV-1 infected male's semen [29]. Examining these increased factors as a whole, however, and not as single entities, provides a network of interplay that would further enhance HIV-1 transmission in the female (Fig. 2). Increased levels of IL-6 and IL-8 will induce recruitment of more lymphoid cells by acting through the FRT. Both cytokines, when interacting with epithelial cells induce inflammation, and therefore recruitment to the site of their interaction.

Because these cytokines are signs of inflammation, the female will increase chemotaxis of neutrophils [116]. At the same time, the increased numbers of neutrophils will begin to differentiate into T lymphocytes due to increased levels of IL-2 being produced by the interaction between the FRT and IL-1α . Because there is an increase in IL-1α concentration in HIV-1 infected semen, there will be more IL-2 produced by the epithelium as a response, thus promoting T helper cell subsets. The Increased levels in IL-16 in the FRT will further enhance the recruitment of $CD4^+$ T lymphocytes to the site of inflammation perhaps by interacting with the interstitially placed T lymphocytes in the ectocervical epithelium [117]. Increased TGF-β levels in semen serve a dual purpose: further dampening of the innate immune response and, in doing so, enhancing the recruitment of T helper cells to the site of inflammation [4]. Interactions between the epithelium and TGF-β will release more TGF-β in the basolateral direction as well as induce IL-16 release [4]. Working together, TGF-β and IL-16 will induce a T_h17 phenotype of some T lymphocytes. As discussed earlier, $CD4^+$ T_h17 lymphocytes were the first immune cells to be infected by HIV-1 [109, 110], therefore increasing their overall numbers to the site of inflammation may further enhance HIV-1 transmission. Most of, if not all, the increased cytokines in HIV-1 infected male's semen increase $CD3^+$ T lymphocyte recruitment to the site of inflammation (Fig. 2). IL-7 may be connected by, in a way, "trapping" the T lymphocytes in the inflamed area by preventing T lymphocyte depletion through apoptosis [94]. In fact, a study revealed that increased IL-7, in conjunction with HIV-1, may help the expanded pool of T

lymphocytes from being depleted and therefore keep the founder pool of HIV-1 infected cells alive for further expansion [94].

Increased levels of the CC chemokine RANTES are particularly interesting in that RANTES may interact with the interstitially found Langherans cells. RANTES induced chemokines through DCs include macrophage inflammatory protein 2 (MIP-2), MIP-1α, MIP-1β, IL-6, and TNF-α [13, 118, 119]. As stated before these cytokines are very much involved in the recruitment, expansion, and activation of $CD4^+$ T lymphocytes [119]. Therefore, it stands to say that HIV-1 infected semen is focused on expanding and activating the $CD4^+$ T cell subsets. In doing so, the induction of a tolerogenic state may be reached quicker than with healthy semen, allowing the virus to persist for longer periods of time.

3.4 Conclusions

Semen is a complex medium that can rapidly manipulate the female innate immune response post-coitus [29, 77] . Manipulation of the FRT immune response functions, most broadly, to prime the female for fertility and reproduction. Evidence of this is the fact that semen elicits the FRT to produce large quantities of IL-8 and TGF-β, which are both key players in angiogenesis and reproduction, respectively. However, in causing the innate immune response to switch from a pro-inflammatory state to a tolerant state, semen may inadvertently increase a female's risk to further pathogen infection, such as HIV-1. By recruiting HIV-1 specific target cells to the site of semen deposition, specifically the transformation zone, the introduction of semen may cause an increased risk of transmission.

However, this is all in the context of healthy male semen. As noted HIV-1 infected male semen contains some of the same factors as healthy male semen, except in varying concentrations with certain major cytokines present in higher quantities [29]. These higher quantities, as explained before, may have downstream implications on the immune cells present in the FRT, which will have downstream effects on how the innate immune response is manipulated. Conversely, some factors may be decreased in concentration and therefore lead to their own immune remodeling [29]. This change in the biphasic nature of semen in the FRT will further enhance HIV-1 transmission. This may very well go all the way back to how HIV-1 manipulates the contents of semen in males before the semen acts on any genital tract, be it male or female, which may be an evolutionary advantage selected for in HIV-1 variants transmitted throughout the course of HIV-1 infections.

More information and studies are needed that take into account every step from semen creation in the male to ending immune cell populations in the female and everything in between. The constituents in semen have profound effects for the FRT, which responds with its own immunodulatory factors. These released factors then impact the immune cells present in the subepithelium of the FRT. Knowing what factors are in semen and how these factors interact with the FRT will provide insights to development of novel therapies. Vaccines to prevent pathogen transmission, as well as treatments for infertility may be developed based on immunological responses seen in the FRT. This review provides a starting point for identifying key aspects of the interaction between semen and the

FRT, which may lead to further analysis of the interaction between semen and the FRT or to the development of said treatments.

Chapter 4

Semen acts through ECT1 epithelial cells to enhance R5 HIV-1 infection of T lymphocytes

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4.1 Abstract

Semen is known to modulate the response of the female reproductive tract (FRT) when both are in contact. Indeed, semen has a physical effect through strengthening tight junctions and thus reducing permeability. Semen also elicits a pro-inflammatory response from the FRT, causing the subepithelial space to become rife with immune cells that further attenuate inflammation. However, what is not known is if the presence of semen on the apical side of the FRT could influence HIV-1 infection in $CD4^+$ T on the basolateral side of the FRT. To elucidate this, we have updated our transwell model system to include a co-culture of primary blood mononuclear cells (PBMCs). By doing so we can see preliminary results of semen's transduced effect through the epithelial barrier. We hypothesize that semen will modulate human immunodeficiency virus type 1 (HIV-1) target cells ($CD4^+$ T cells) to become more susceptible to HIV-1. To extend this idea further, we wanted to also garner answers to the bottleneck hypothesis, which states that HIV-1 transmission in discordant, heterosexual couples is almost always R5-tropic driven; using CD4 and CCR5 as co-receptors over CD4 and CXCR4. Our results indicate that the presence of semen on the apical side of an ECT1 epithelium had a rescue effect of CCR5 on $CD4^+$ T cells in basolateral compartment while not affecting CXCR4. When the PBMCs were challenged with a pseudovirus infection using a R5 or X4 envelope, viral infection and gene expression with a R5-tropic pseudovirus were enhanced in the presence of semen. This leads us to conclude that semen plays a significant role in the bottleneck hypothesis.

4.2 Introduction

Studies that stemmed from identifying components in semen associated with infertility have begun characterizing semen [9, 15, 35, 42, 49, 59, 74, 120]. Semen is no longer thought of as an inert vehicle for sperm or pathogen delivery, but as a biologically active substance [9, 16, 29, 62, 70, 84, 99, 121-123]. The constituents of semen; sugars, whole cells, cytokines, proteins, etc. have been shown to interact with tissues they come in contact with [9, 28, 29, 85, 99, 106, 121, 124] (Appendix Chapters 1 and 2). Previous work of ours has identified calcium and proteins in semen as potent activators of tight junctions in cell lines derived from the female reproductive tract (FRT) (Appendix Chapter 2). Semen does not just induce mechanical alterations to reproductive epithelia, it also elicits an immunological response, which we have previously reported as being pro-inflammatory (Appendix Chapter 1).

Indeed, when semen interacts with the FRT it triggers the innate immune response mounted by the female [95, 99, 100]. The initial inflammation associated with the innate immune response is pivotal in identifying paternal MHC Class I and II receptors of abnormal or non-functional spermatozoa [9, 29, 99, 104]. However, inflammation must soon be abated or all spermatozoa, including healthy, viable sperm, will be cleared. Therefore, in order to ensure the survival of healthy sperm, semen modulates the innate immune response to go from inflammation to tolerance [70, 76, 95, 99, 125, 126]. A tolergenic state in the FRT will allow for semen, and its constituents, to initiate the early stages of pregnancy and embryo development.

Induction of tolerance is carried out quickly when semen is deposited in the FRT [99]. Early induction of T regulatory cells (Tregs) have been shown in animal models and the generation of Tregs dampen the immune response to paternal antigens [28, 124]. The remodeling of the female immune system is paramount to pregnancy but also has consequences downstream of sperm survival. Indeed, the interaction of constituents like transforming growth factor beta (TGF-β) with reproductive epithelia release cytokines like tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) to the subepithelial space [4, 9, 29]. Cytokines like these have lasting impacts on immune cells naturally found beneath, and interstitially to, the epithelia. Release of chemokines from the epithelium brings about an influx of immune cells from peripheral blood such as monocytes, macrophages, dendritic cells (DCs), natural killer cells (NKs), B cells, and T lymphocytes. Each immune cell recruited to the site of inflammation adds their own effect to the immune cascade, which can include further immune cell chemotaxis, maturation of naïve or immature immune cells, or adding immune cell-specific cytokines to the milieu [95, 126, 127]. In fact, semen deposition in the FRT has been shown to increase immune cell concentrations in the ectocervix, the lower portion of the cervix, through chemoattractant molecules [103, 104].

The ectocervix represents the transformation zone, which is the portion of the FRT that contains the highest concentration of all immune cells [102-104]. Considered the central hub for cell-mediated immunity, the ectocervix is immunologically very active compared to the rest of the FRT [103]. Therefore, when semen comes in contact with the FRT, the transformation zone will

coordinate the innate immune response mounted against semen [103], i.e. by increasing the concentration of effector T cells, which carry out cell-mediated responses.

However, effector T cells, specifically $CD4^+$ T cells, are of note because of their involvement in human immunodeficiency virus type 1 (HIV-1) transmission. During heterosexual intercourse the chances of a woman acquiring HIV-1 are twice as much as her male counterpart [9, 29]. Perhaps this is why in 2013, 62% of all new HIV-1 infections in adolescents occurred among females [128]. In Ethiopia about 60% of new HIV-1 infections occur in discordant heterosexual couples [129]. In sub-Saharan Africa 58% of people living with an HIV-1infection are women [129]. Therefore, worldwide, women are disproportionately affected by HIV-1.

During heterosexual transmission of HIV-1, studies have pinpointed specific genetic variants as being predominant isolates that initiate infection in females [130, 131]. Known as R5-tropic viruses, they establish infection early on during transmission and can lead to systemic infection [130-132]. Often, tracing back the genetic isolate that started infection leads to R5-tropic viruses, even down to a single R5-tropic virion [131]. It is known that HIV-1 needs to come in contact with a CD4 receptor on a cell to initiate entry, however the virus also needs a co-receptor; either CCR5 or CXCR4. Depending on which co-receptor the virus utilizes with CD4 it is termed either R5 (CCR5-utilizing) or X4 (CXCR4-utilizing). Despite infection being initiated by primarily R5-tropic viruses both viral types may be present during transmission events and can both initiate infection [132].

However, it is still unknown why R5-tropic viruses are, almost exclusively, the virus that initiates infection in a female.

Studies attempting to understand the R5-tropic bias of HIV-1 transmission from male-to-female have identified key aspects affecting genetic variants of HIV-1, collectively called the bottleneck hypothesis [130-132] . Indeed, the bottleneck hypothesis posits that there are various filtering points that select out for specific genetic variants [132]. These checkpoints exist from the male reproductive tract all way through semen deposition in the FRT. At each step along the way the bottlenecks select for the advancement of specific variants through many biological factors, however the most stringent bottleneck is when semen is introduced in the FRT [131, 132].

Therefore, using a previously established *in vitro* transwell system as an analog of the polarized topography of the FRT epithelium (Appendix Chapters 1 and 2) we aimed to elucidate if the response of the FRT to semen would favor infection of one HIV-1-tropic strain over the other. The transwell model system used an ectocervical cell line derived from the FRT [133] that was grown in a polarized manner on a mesh insert, which allowed the independent sampling of both the apical and basolateral chamber. Literature suggests that the ectocervix is the site of HIV-1 infection during male-to-female transmission [9, 29, 95, 103, 113, 131]. Thus, in this study we used the ectocervix derived cell line ECT1/E6E7 (ECT1). By advancing the model system to incorporate peripheral blood mononuclear cells (PBMCs) in the basolateral chamber, we can elucidate if semen

transduces its effect across the epithelium to enhance the infectivity of one HIV-1-tropic strain over the other.

Indeed, by co-culturing PBMCs with ECT1 cells it was found that semen elucidated factors to be released from the epithelium that interacted with peripheral blood cells, which induced an immunological environment that enhanced infections by both R5- and X4-tropic viruses. However, the fold-change of $CD4^+$ T cells that were infected by R5-tropic viruses in the presence of semen compared to those in the absence of semen was higher than X4-tropic viruses. These data suggest that the interaction between semen and the epithelium is enough to drive R5-tropic infection during HIV-1 transmission from male-to-female.

4.3 Materials and Methods

4.3.1 Cell line maintenance

Experiments were performed using a cell line derived from one of the three regions of the human female reproductive tract: ectocervical cells (ECT1/E6E7) [133].

Prior to seeding, cells were grown and maintained in T150 tissue culture flasks (Grenier Bio One, Monroe, NC) at 37°C with 5% CO_2. ECT1 cells were cultured using the Keratinocyte Serum-Free Medium (Gibco, Grand Island, NY), which included 50 µg/ml bovine pituitary extract (BPE), 0.1 ng/ml epithelial growth factor (EGF), 50X penicillin/streptomycin solution, and with the addition of 0.4 mM calcium chloride (EMD Millipore, Billerica, MA).

4.3.2 Transwell culture system

ECT1 cells were cultured as a confluent, polarized monolayer by seeding into the apical chamber of a 6.5 mm, 0.4 µm pore size, polyester (PET) transwell insert in a 24-well receiver plate (Corning, Corning, NY). 200 µl of 1.5 x 10^5 cells were placed in the apical chamber, with 600 µl of media in the basolateral chamber. Time to confluence varied between 14 and 17 days post-seeding. TEER readings were taken every other day to testthe growth and potential of the plate.

4.3.3 Semen

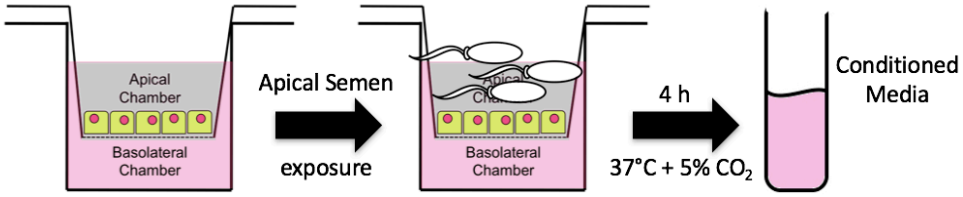
Semen was purchased as 20 ml of pooled semen from a mixture of ≥3 healthy donors between 26 and 34 years of age (Lee Biosolutions, St. Louis, MO). Specimens were shipped overnight on dry ice and immediately divided into 500 µl aliquots and frozen at -20°C upon arrival. In all descriptions, semen refers to whole semen; containing sperm.

4.3.4 Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were obtained from Comprehensive NeuroAIDS Center (CNAC) Mammalian Cell and Virus Core cohort at Temple University. The PBMCs were then cultured overnight in RPM1-1640 supplemented with 2% (10 ml) heat-inactivated FBS and 1% (5 ml) HEPEs (R1H medium). Additional interleukin 2 (10 U/ml) was added to the medium for overnight activation. After 16-18 h incubation the cells were then spun down at 2500 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in the appropriate amount of R1H to reach a concentration of 1 x 10^6 cells/ml. Unless stated otherwise PBMCs were seeded at 1 x 10^6 cells/ml.

4.3.5 Conditioned media collection of semen exposed ECT1 cells

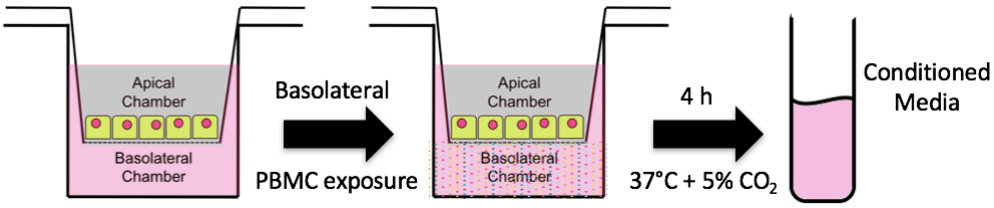
Transwells of confluent ECT1 monolayers had both their apical and basolateral chambers aspirated and then were washed with 1X HBSS twice. The basolateral chamber was then re-supplemented with 600 µl of media. The apical chamber was then supplemented with 25% semen in duplicate for 4 h. 4 h was chosen because this time is consistent with the literature to be the time it would take for semen to have an interaction with the epithelial tissue and be cleared from the FRT. During the 4 h the plates were incubated at 37°C + 5% CO_2. After 4 h the basolateral chamber media was collected and stored at -80°C. Below is a schematic representation of conditioned media collection.



4.3.6 Collection of conditioned media of ECT1 cells co-cultured with PBMCs

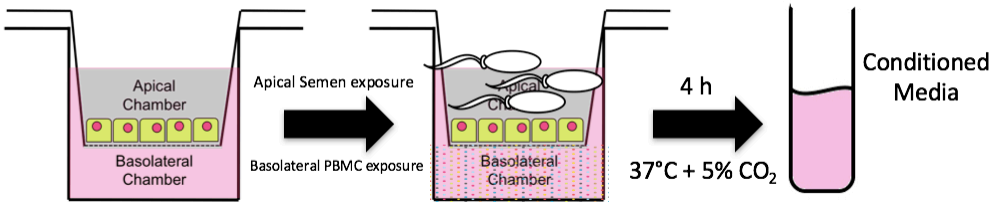
Transwells of ECT1 monolayers at confluency had both their apical and basolateral media aspirated and discarded. Each well was washed twice with 1X HBSS. The basolateral chamber was then supplemented with 600 µl of 1 million IL-2 activated PBMCs. The ECT1 cells and PBMCs were in co-culture for 4 h and incubated at 37°C + 5% CO_2. Again, 4 h was chosen because it is an accepted time frame, in the literature, for the innate immune response to have been initiated and taking effect. After 4 h the conditioned media were harvested by collecting the basolateral chamber media and PBMCs and then spinning down the sample at

2500 rpm for 5 min. The supernatant was placed into a separate tube and stored at -80°C. The PBMC pellet was resuspended in 500 µl R1H medium for further testing using flow cytometry. Below is a schematic representation of conditioned media collection.



4.3.7 Conditioned media collection of semen exposed ECT1 cells co-cultured with PBMCs

Transwells of ECT1 monolayers had both the apical and basolateral chambers aspirated and discarded. Each well was washed twice with 1X HBSS. The basolateral chamber was supplemented with 600 µl of 1 million IL-2 activated PBMCs while the apical chamber had 200 µl of 25% semen added to it. The transwells were incubated at 37°C + 5% CO_2 for 4 h. After 4 h the basolateral chambers were harvested and spun down at 2500 rpm for 5 min. The supernatant was collected and stored at -80°C while the PBMC pellet was resuspended in 500 µl of R1H medium and kept for flow cytometry analysis.



4.3.8 ELISArray

The collected supernatants were analyzed by the Qiagen Human TLR-induced Cytokines II: Microbial-induced Multi-Analyte ELISArray Kit (Qiagen,

Maryland). As per the protocol conditioned media was added to each well of a pre-coated 96-well plate with 12 different antibodies. After incubation and washing three times, a detection antibody was added. After incubation and washing three times, Avidin-HRP was added to each well. After another incubation and washing 4 times, a development solution was added to each well. After a 15 minute incubation, in the dark, a stop solution was added to each well. The plate was then read on a spectrophotometer at OD450 with wavelength correction at OD570. The results were analyzed in Microsoft Excel.

4.3.9 Flow Cytometry

PBMCs collected from transwells of ECT1 cells apically exposed to 25% semen were spun down for conditioned media (supernatant) collection. The cells were then resuspended in R1H medium for antibody staining for Flow Cytometry. 5 µl of anti-CCR5 antibody conjugated to PE (eBioscience, San Diego, CA) was applied first and left to incubate for 45 minutes at room temperature (25ºC). The cells were then washed with 2 ml of R1H and spun down at 2500 rpm for 5 minutes. The PBMCs were then resuspended in a volume of R1H medium for further staining. 5 µl of anti-CD4 conjugated to APC (eBioscience, San Diego, CA), 5 µl of anti-CXCR4 conjugated to BV 421 (BioLegend, San Diego, CA), and 3 µl of anti-CD3 conjugated to APC-Cy7 (BioLegend, San Diego, CA) were then added to each tube. The antibodies were left to incubate for 40 minutes at 4ºC. After 40 minutes, 1 µl of a Live/Dead stain, conjugated to BV 510 (AmCyan or Aqua) (eBioscience, San Diego, CA) was applied to each tube. The final solution was left to incubate at 4ºC for another 15 minutes. After total incubation the PBMCs were

washed with 2 ml R1H medium and spun down at 2500 rpm for 5 minutes. The cells were resuspended in 500 µl of 1% paraformaldehyde (PFA) for 40 minutes at room temp. After incubation the cells were washed with 2 ml R1H medium and spun down at 2500 rpm for 5 minutes. After this final spin the cells were resuspended in 500 µl of a filter sterilized buffer consisting of 500 ml of HBSS supplemented with 3% (15 ml) heat-inactivated FBS, 2.5 mM (1.25 ml of a 1 M solution) of $CaCl_2$, and 0.02% (0.1 g) sodium azide (Flow/Facs Buffer). The cells were then analyzed via flow cytometry on a LSR Fortessa. All data were then analyzed in FlowJo software (version 10.1r5) and Microsoft Excel.

4.3.10 Pseudovirus Propagation

Pseudovirus was created using a copy of the HIV-1 backbone plasmid pNL4.3-ΔE-EGFP (NIH AIDS Reagent Program, Bethesda, MD) and a copy of the envelopes HXB2 and a previously identified Transmitted Founder envelope (T/F) (NIH AIDS Reagent Program Reagent p1012.TC21.3257). The HIV-1 backbone plasmid and one of the envelope plasmids were co-transfected into HEK 293 cells (ATCC, Manassas, VA). Pseudovirus produced will be in the supernatant, therefore the supernatant was harvested and kept at -80ºC until use.

4.3.11 Pseudovius Infections

Pseudovirus (PSV) with either HXB2 (X4 Virus) or T/F (R5 Virus) envelopes were used in transmission studies. Immediately after a 4 h 25% semen exposure each transwell insert was removed. In the exposed basolateral chamber 600 µl of diluted PSV of either envelope (1:10 for X4 envelope PSV and 1:4 for R5 envelope PSV) type was added, but no more than one type per well. The infection took place

for 4 h. After 4 h the samples were collected and spun down at 2500 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 500 µl of R1H medium. They were then placed back in the 37ºC incubator + 5% CO_2 for 48 h. After such time the samples were spun down at 2500 rpm for 5 minutes. With the supernatant discarded, the PBMCs were then resuspended in R1H medium for staining. 5 µl of anti-CD4 conjugated to APC (eBioscience, San Diego, CA) and 3 µl of anti-CD3 conjugated to APC-Cy7 (BioLgened, San Diego, CA) was added to each tube. After incubating for 40 minutes, a Live/Dead stain conjugated to BV 521 (eBioscience, San Diego, CA) was added and the tubes re-incubated for another 15 minutes. After such time, the tubes were washed with 2 ml R1H medium and spun down at 2500 rpm for 5 minutes. The PBMCs were then resuspended in 500 µl of 1% PFA for 40 minutes. After incubation the cells were washed and spun down as noted before and resuspended in 500 µl of Flow/Facs Buffer and immediately assayed on a LSR Fortessa flow cytometer. All data were analyzed in FlowJo software and Microsoft Excel.

4.3.12 Statistical analyses

p-Values were calculated for the difference between pairs of treatments conditions using a two-tailed Student's *t*-test, and a value of less than 0.05 was the limit for statistical significance. p-Values are indicated on the figures and within the figure legends.

4.4 Results

4.4.1 Semen initiated a pro-inflammatory environment from ECT1 cells

Our purpose was to introduce subepithelial immune cell populations into our transwell model of the FRT for the purpose of examining the combined responses of epithelial keratinocytes and immune cells to the apical application of semen. In these first experiments we examined the output of soluble immunomodulatory factors in the basolateral chamber after application of semen. After semen exposure basolateral chambers from transwells were collected and assayed for a panel of 12 pro-inflammatory cytokines. The concentrations were then expressed as a fold-change over mock conditions, which were wells that were not apically exposed to semen. These data served as a base line for the effect of semen on ECT1 cells so when PBMCs are incorporated into the system the combined effect can be further evaluated.

Semen elicited a pro-inflammatory response, which included release of select pro-inflammatory cytokines TNF-α, interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 12 (IL-12), interleukin 17A (IL-17A), interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), regulated on activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein 1 alpha and 1 beta (MIP-1 α and MIP-1β), macrophage derived chemokine (MDC), and Eotaxin (Fig. 4.1). Most of the cytokines had about a 1-fold change increase in concentration over mock conditions (Fig. 4.1A). IL-8 concentration had about a 3-fold increase over mock, which was the largest change in concentration (Fig. 4.1A). The next largest fold-change in concentration came from Eotaxin and MCP-1 with

1.19- and 1.15-fold change over mock, respectively. IL-17A had the lowest concentration with a 0.89-fold change over mock. Therefore, almost every analyte, with the exception of IL-8, was in the range of about 0.9-1.2-fold change over mock. These data suggest that exposure to semen to ECT1 cells elicited a pro-inflammatory environment in the basolateral chamber that was replete with chemokines and chemoattractant factors.

4.4.2 PBMCs co-cultured with ECT1 cells attenuated inflammation to a higher degree

To establish a baseline for the effect PBMCs had on immunomodulatory factor release from the ECT1 epithelium we co-cultured primary human immune cells in the basolateral chamber of transwells that had ECT1 cells grown as a polarized monolayer on the mesh insert. Fresh PBMCs were first grown overnight in media containing additional interleukin 2 (IL-2) to ensure the survival of T lymphocytes. The next day the PBMCs were seeded into the basolateral chambers of some transwells. Some wells were devoid of PBMCs to serve as a mock, termed control wells. After a 4 h co-culture the basolateral chamber was collected and the supernatants were assayed for the panel of 12 cytokines. These data represent the cross-talk between peripheral blood cells and the ECT1 epithelium, which will act a baseline for the next set of experiments. All concentrations are expressed as a fold-change of PBMC exposed wells over control wells.

Figure 4.1A

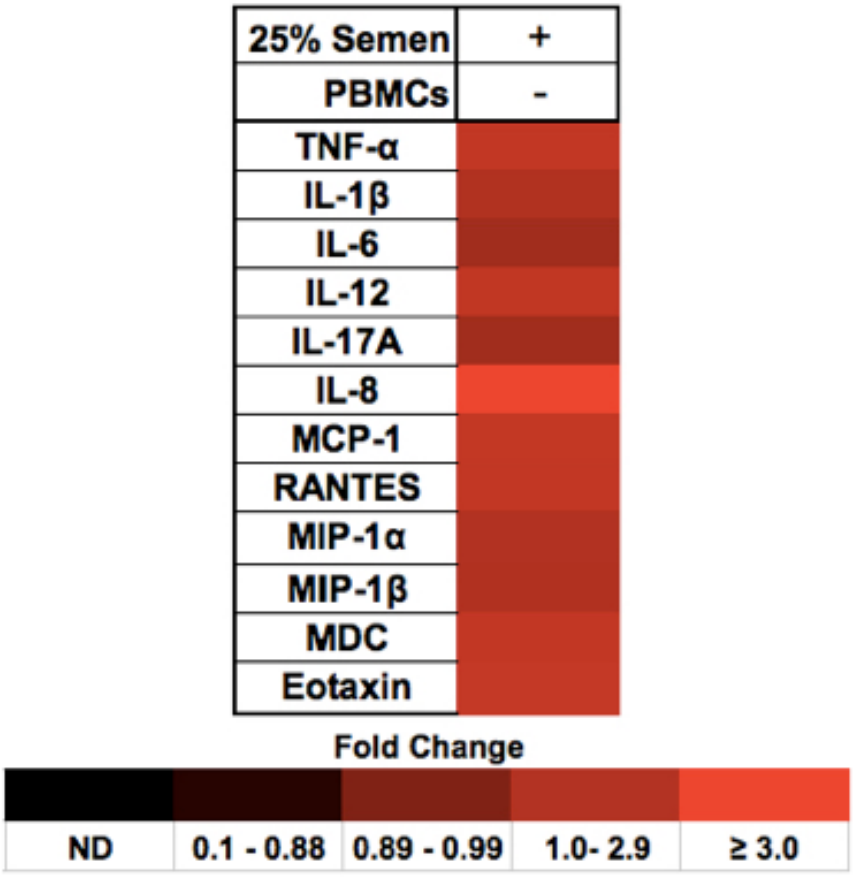

25% Semen
+
PBMCs
-
TNF-α
IL-1β
IL-6
IL-12
IL-17A
IL-8
MCP-1
RANTES
MIP-1α
MIP-1β
MDC
Eotaxin
Fold Change
ND
0.1 - 0.88
0.89 - 0.99
1.0- 2.9
≥ 3.0

Figure 4.1B

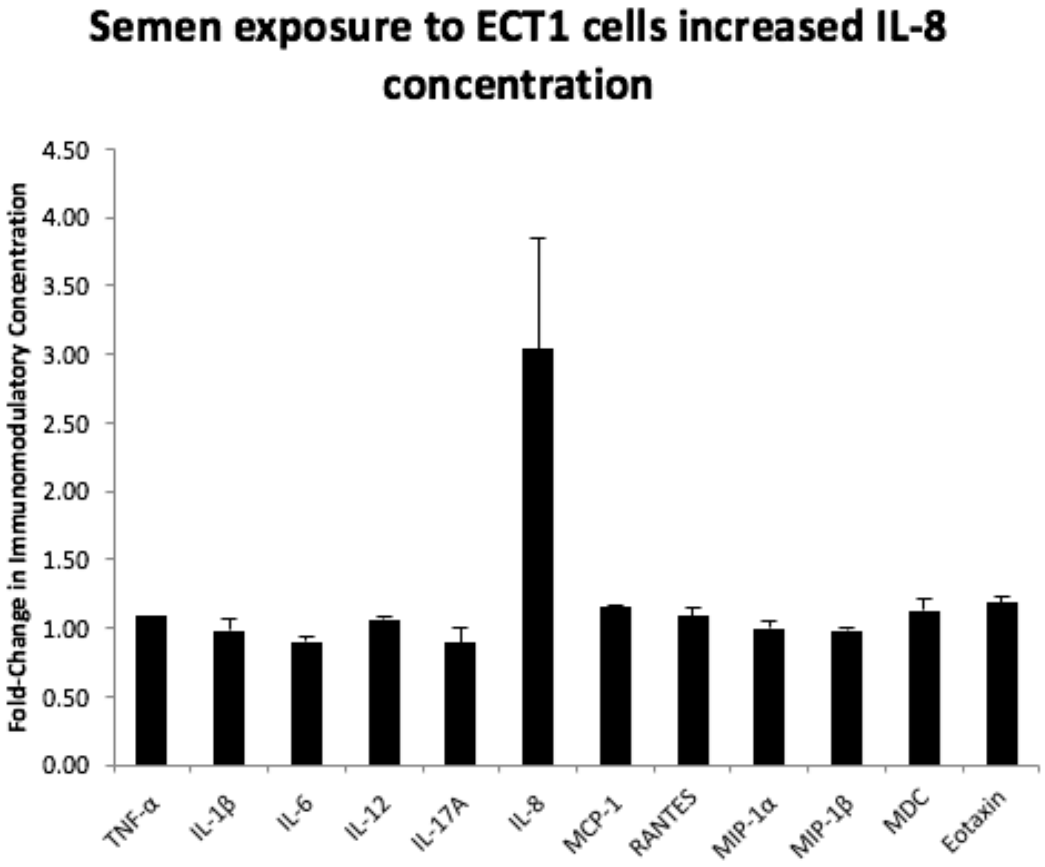

Semen exposure to ECT1 cells increased IL-8 concentration
Fold-Change in Immunomodulatory Concentration
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
TNF-α
IL-1β
IL-6
IL-12
IL-17A
IL-8
MCP-1
RANTES
MIP-1α
MIP-1β
MDC
Eotaxin

Figure 4.1C

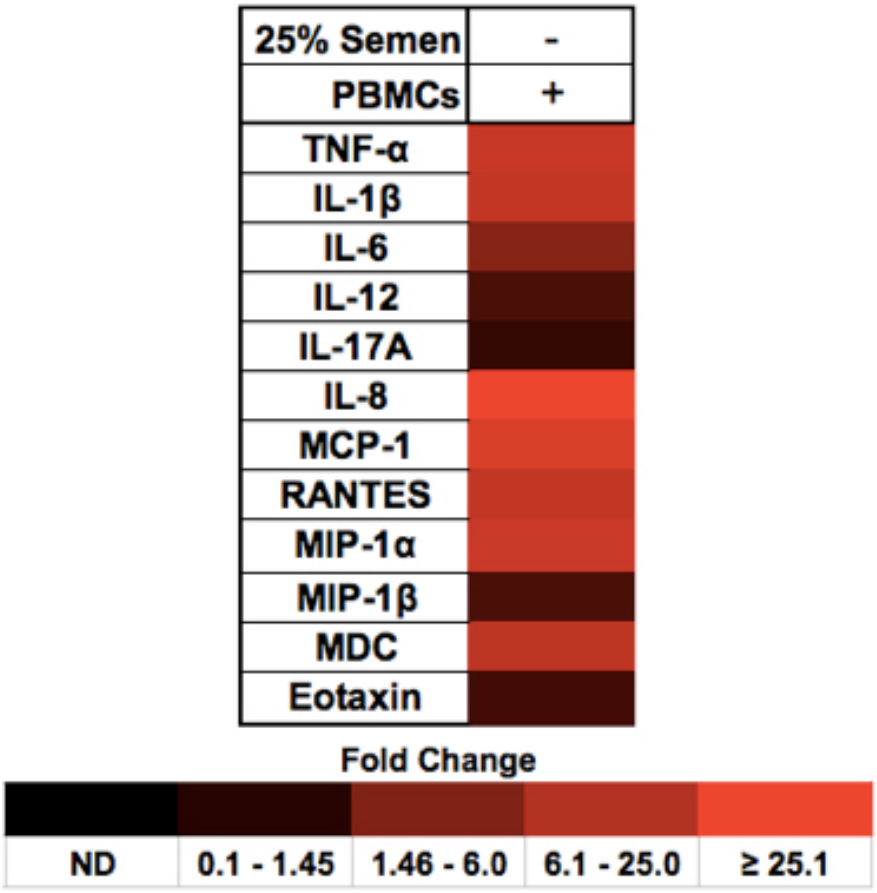

25% Semen
-
PBMCs
+
TNF-α
IL-1β
IL-6
IL-12
IL-17A
IL-8
MCP-1
RANTES
MIP-1α
MIP-1β
MDC
Eotaxin
Fold Change
ND
0.1 - 1.45
1.46 - 6.0
6.1 - 25.0
≥ 25.1

Figure 4.1D

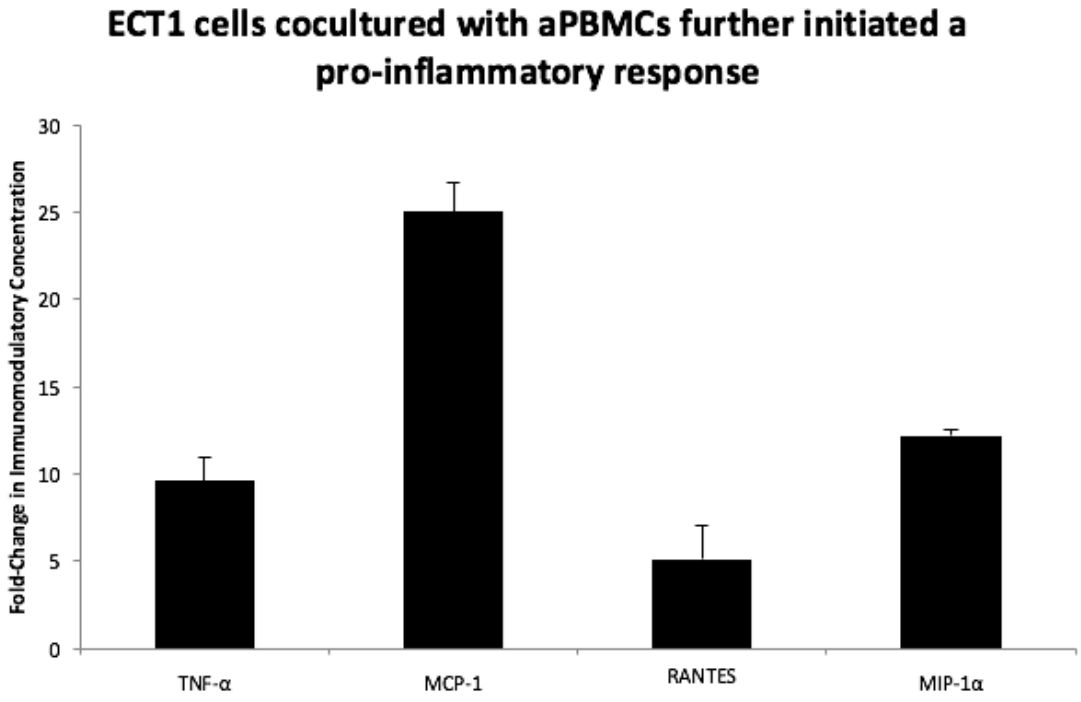

ECT1 cells cocultured with aPBMCs further initiated a pro-inflammatory response
Fold-Change in Immunomodulatory Concentration
30
25
20
15
10
5
0
TNF-α
MCP-1
RANTES
MIP-1α

Figure 4.1E

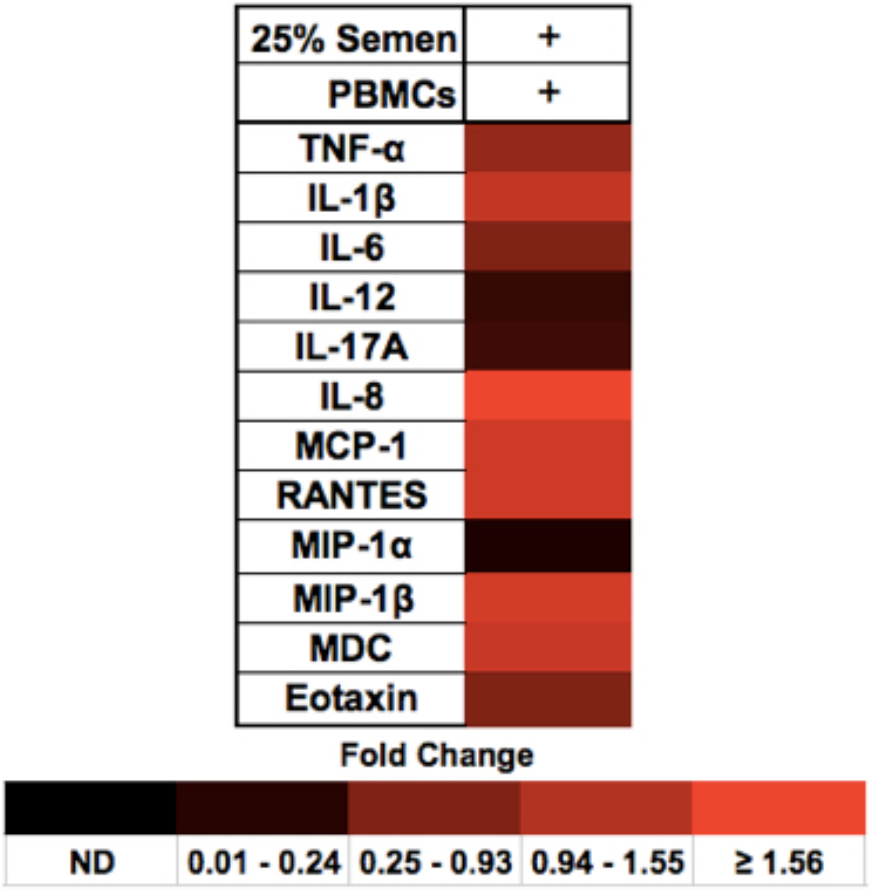

25% Semen
+
PBMCs
+
TNF-α
IL-1β
IL-6
IL-12
IL-17A
IL-8
MCP-1
RANTES
MIP-1α
MIP-1β
MDC
Eotaxin
Fold Change
ND
0.01 - 0.24
0.25 - 0.93
0.94 - 1.55
≥ 1.56

Figure 4.1F

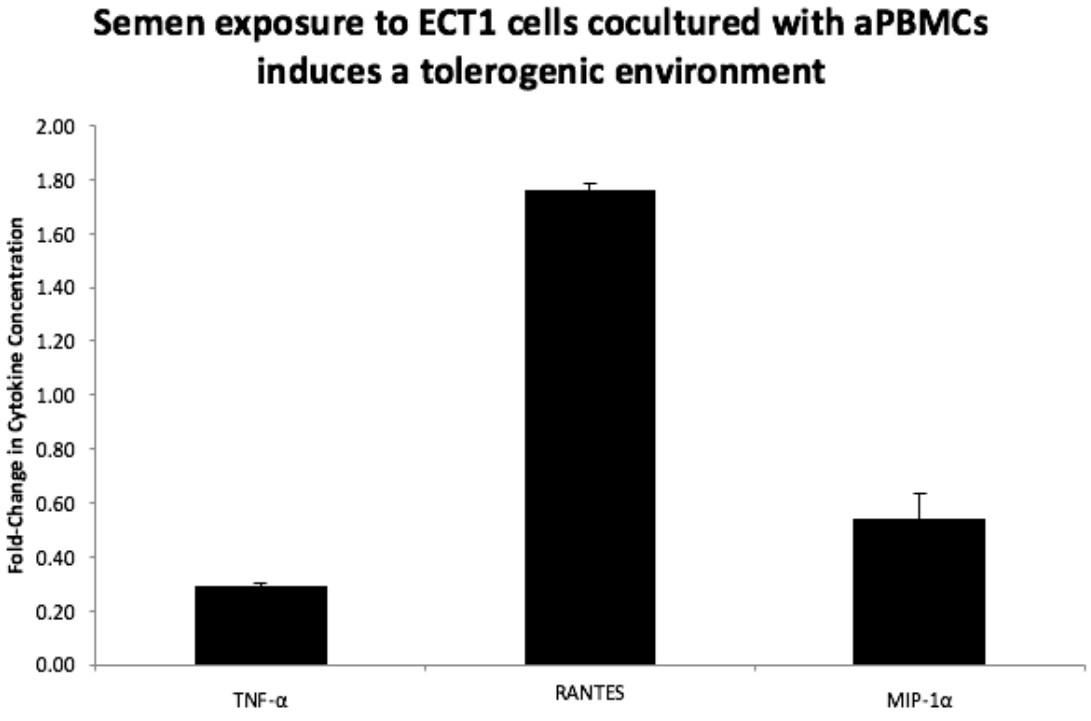

Semen exposure to ECT1 cells cocultured with aPBMCs induces a tolerogenic environment
Fold-Change in Cytokine Concentration
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
TNF-α
RANTES
MIP-1α

Fig. 4.1. The ectocervical epithelium releases soluble immunomodulatory factors basolaterally in response to 25% semen and PBMCs treatment. Changes in factor concentrations in the basolateral chambers are shown as a heat map of fold-change over conditions of 12 analytes detected in conditioned media collected from the basolateral supernatants at 4 h after epithelial exposure to either (A) 25% semen, (C) PBMCs, or (E) both together. (B) Analytes released basolaterally in response to 25% semen were predominated by IL-8. (D) Analytes released basolaterally in response to co-culture with PBMCs included the pro-inflammatory cytokines TNF-α, MCP-1, RANTES, and MIP-1α. (F) After 4 h epithelial exposure to both 25% semen and PBMCs analyte concentrations of TNF-α and MIP-1α were reduced, however RANTES concentration was slightly increased. Semen had a focused, direct effect of these analytes when compared to PBMCs alone.

When PBMCs were co-cultured with the ECT1 epithelium, every analyte was increased by at least 1.5-fold (Fig. 4.1E). On the lower end of modulation, IL-12, IL-17A, MIP-1β, and Eotaxin were present with 1.95-, 1.44-, 1.93-, and 1.74-fold changes over control wells (Fig. 4.1E). IL-17A, again, had the lowest fold-change over mock conditions like it did during the semen only experiments. The slight increase in IL-12 concentration may account for the reason why there was a 9.62-fold increase over control wells in TNF-α concentration (Fig. 4.1E). IL-12 is an inducer of TNF-α from T cells to further extend inflammation [134] and like IL-12, IL-17A regulates IL-6 expression from T cells [104]. Perhaps the 1.44-fold change over control wells in IL-17A could explain the almost 3.5-fold increase over control wells in IL-6 (Fig. 4.1C).

Interestingly, MIP-1β was increased in concentration about 2.0-fold over control wells (Fig. 4.1C). However, MIP-1β is one form of macrophage inflammatory protein, with the other being MIP-1α. MIP-1α, in this experiment, had a large increase in concentration over control wells with close to a 12-fold increase (Fig. 4.1C). Both forms of macrophage inflammatory protein induce the release of TNF-α and IL-6, which, again, may explain their increased concentrations.

Of note, there was a greater than 25-fold increase in MCP-1 concentration over control wells (Fig. 4.1C). However, the largest fold-change over control wells was IL-8 with about a 58-fold increase (Fig. 4.1C).

Looking at the data as a whole, ECT1 cells, and the peripheral blood cells they were co-cultured with, generated a very pro-inflammatory environment replete with large increases in factor concentrations involved in cellular recruitment.

4.4.3 Semen exposure further increased MCP-1, RANTES, and MIP-1β concentrations in transwells of ECT1 cells co-cultured with PBMCs

The macrophage inflammatory proteins highlight the fact that immune cell released factors, like MIP-1α and MIP-1β, recycle back to other peripheral blood cells to induce the release of other cytokines. These released cytokines further interact with other peripheral blood cells as well as the ECT1 epithelium to induce the release of more chemokines. Therefore, establishing the baseline of the interplay between PBMCs and ECT1 cells was paramount to identifying semen-specific changes during co-culture of PBMCs and semen exposure. Our purpose with these set of data was to identify what factors are released from the ECT1 epithelium, above and beyond what is normally released due to the interplay of peripheral blood cells and ECT1 cells. This data informed on how semen modulates an already pro-inflammatory environment.

Transwells containing confluent monolayers of ECT1 cells grown on the mesh insert were basolaterally supplemented with IL-2 treated PBMCs just before semen exposure as in the other experiment. However, in this case some wells were apically exposed to semen. After 4 h, basolateral chambers from all transwells were collected and the supernatant was assayed for cytokine concentration changes between variables. Cytokine concentrations are expressed as fold-changes of semen exposed wells over mock, termed non-semen exposed wells.

As with the other conditions, IL-8 had the largest increase in concentration with almost a 5-fold change over non-semen exposed wells (Fig. 4.1E). This was

in addition the large, 58-fold change over control wells associated with just PBMCs alone (Fig. 4.1C). Indeed, the fold-changes of analytes in this experiment (Fig. 4.1E) are all in addition to the fold-changes obtained when peripheral blood cells are co-cultured with just ECT1 cells alone (Fig. 4.1C).

Therefore, analytes like TNF-α, IL-1b, IL-6, IL-12, IL-17A, MIP-1α, and Eotaxin all had about the same concentration in semen exposed co-cultured wells as they did in non-semen exposed co-cultured wells with fold-changes of 0.29-, 0.47-, 0.25-, 0.11-, 0.12-, 0.05-, and 0.25-fold changes over non-semen exposed wells, respectively (Fig. 4.1E). Semen did not further increase their release from either the epithelium or immune cells. However, TNF-α, IL-1b, IL-6, and MIP-1α are already increased 9.62-, 5.92-, 3.56-, and 12.19-fold over control wells (Fig. 4.1C). Thus, even though the analyte concentrations did not seem to be further enhanced by semen exposure their relative concentrations were already high due to the co-culture of PBMCs and ECT1 cells. α

MCP-1, RANTES, and MIP-1B all had further increases due to semen with 1.14-, 1.19-, and 1.54-fold changes over non-semen exposed wells (Fig. 4.1E). MCP-1, when measured against its concentration at base level, was still over 25-fold increased due to semen (Figs. 1B and 1C). RANTES and MIP-1β, when examined in the same way, were now at 6- and 3.5-fold higher concentrations (Figs. 1B and 1C). Therefore, semen increased MCP-1, RANTES, and MIP-1β release from either the ECT1 epithelium or PBMCs co-cultured with the epithelium.

4.4.4 Semen exposed or non-semen exposed $CD3^+CD4^+$ T cells co-cultured with ECT1 cells do not differ in relative abundance or CD4 surface expression

Our first purpose was to identify what type of cytokines were produced from ECT1 cells co-cultured with PBMCs during semen exposure. In doing so we identified key cytokines that may further modulate the peripheral blood cells. Therefore, the next set of experiments were aimed at identifying if these cytokines had a lasting impact on HIV-1 target cells in such a way that would favor the infection of either HIV-1 tropic viruses over the other. In order to obtain these data PBMCs co-cultured with ECT1 cells either exposed to semen or not were harvested. The peripheral blood cells were then stained with antibodies for CD3, CD4, CCR5, and CXCR4 conjugated to fluorochromes. Using flow cytometry, the cells were assayed for relative cell numbers and surface expression of each co-receptor. Our thought process is that the cytokine environment will have lasting impacts on co-receptor availability, which will have direct implications on infection by R5- or X4-tropic viruses.

The first step in this process was to evaluate if the ECT1 epithelium and cytokine environment affected $CD3^+CD4^+$ T cells. If $CD3^+CD4^+$ T cells were diminished in these conditions, then both HIV-1 tropic strains would have an equally harder chance at initiating infection due to either the reduced cell numbers of $CD3^+CD4^+$ T cells or the reduced surface expression of CD4. In order to justify the effect of the ECT1 epithelium in the absence of semen, PBMCs grown overnight with IL-2 supplemented media were assayed as well, termed input cells. These data will

provide a background for any effect the ECT1 epithelium has on peripheral blood cells it is co-cultured with.

$CD3^+CD4^+$ T cells comprised 47.75% of all lymphocytes found in input cells (Fig. 4.2B). When compared to PBMCs co-cultured with ECT1 cells, the relative abundances of the total lymphocyte populations were comparable (Fig. 4.2B). $CD3^+CD4^+$ T cells co-cultured without semen comprised 43.63% of total lymphocytes and $CD3^+CD4^+$ T cells co-cultured with ECT1 wells exposed to semen comprised 40.18% of total lymphocytes (Fig. 4.2B). Therefore, the ECT1 epithelium and semen exposure had no effect on the abundance of $CD3^+CD4^+$ T cells found in the total lymphocyte population.

Indeed, the populations of $CD3^+CD4^+$ T cells in each condition, when overlaid on one another on a histogram, show no changes (Fig. 4.2C). Surface expression of CD4 on input $CD3^+CD4^+$ T cells, ECT1 co-cultured $CD3^+CD4^+$ T cells, and semen exposed ECT1 co-cultured $CD3^+CD4^+$ T cells were not statistically different from one another (Fig. 4.2D)

Figure 4.2A

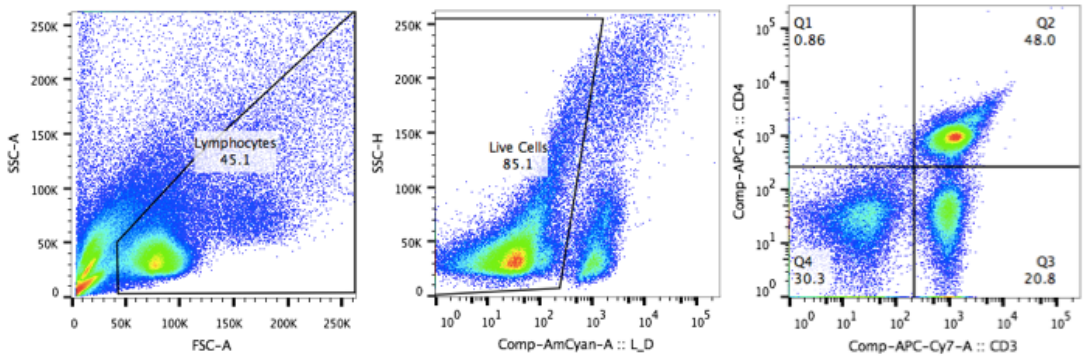

SSC-A
FSC-A
Lymphocytes
45.1
SSC-H
Comp-AmCyan-A :: L_D
Live Cells
85.1
Comp-APC-A :: CD4
Comp-APC-Cy7-A :: CD3
Q1
0.86
Q2
48.0
Q4
30.3
Q3
20.8

Figure 4.2B

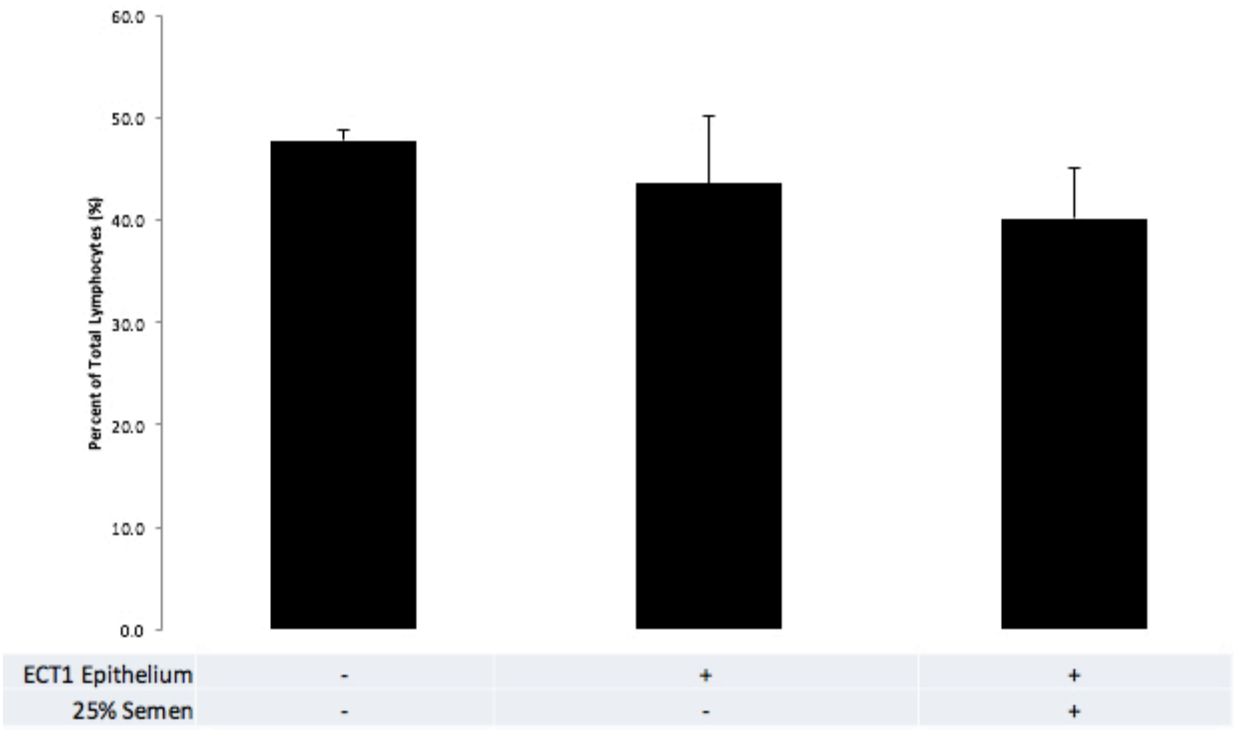

60.0
50.0
40.0
30.0
20.0
10.0
0.0
Percent of Total Lymphocytes (%)
ECT1 Epithelium - + +
25% Semen - - +

Figure 4.2C

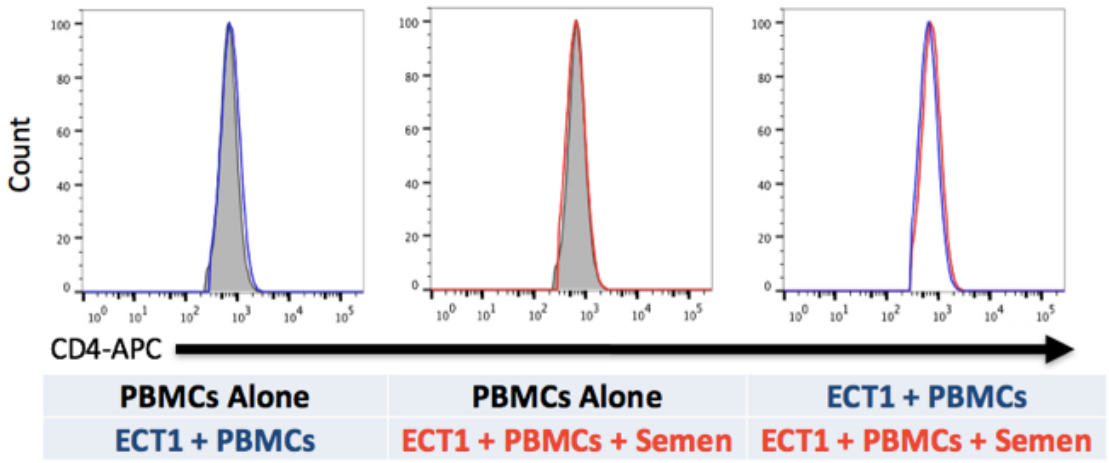
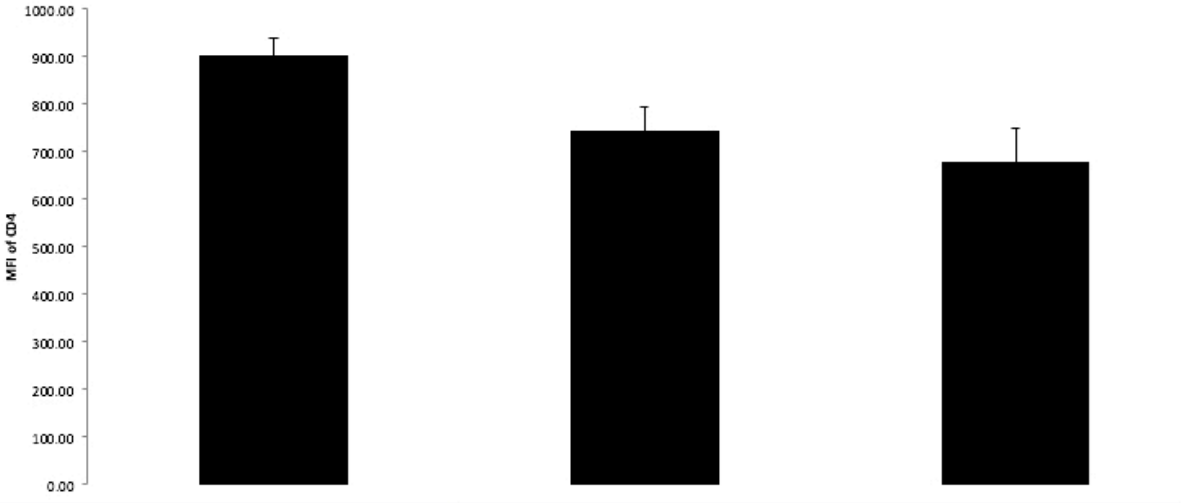

Count
CD4-APC
PBMCs Alone
ECT1 + PBMCs
PBMCs Alone
ECT1 + PBMCs + Semen
ECT1 + PBMCs
ECT1 + PBMCs + Semen

Figure 4.2D



ECT1 Epithelium	-	+	+
25% Semen	-	-	+

Fig. 4.2. Semen does not affect $CD3^+CD4^+$ T cells.

(**A**) PBMCs from transwells were collected at 4 h from the basolateral chambers from mock and semen wells. The number of $CD3^+CD4^+$ T cells is expressed as a percentage of the total Lymphocyte population found in the harvested PBMCs. (**B**) Co-culturing of $CD3^+CD4^+$ T cells with the ECT1 epithelium did not change the population of $CD3^+CD4^+$ T cells. (**C**) and (**D**) There were no population shifts in $CD3^+CD4^+$ T cells during any condition and the surface expression density of CD4 did not change when semen was applied to the system. MFI is median fluorescence intensity; blue is mock treated, red is semen treated, and gray is PBMCs alone without co-culture.

These data suggested that $CD3^+CD4^+$ T cells in each condition were not affected by culturing conditions.

4.4.5 Semen elicits a protective effect on $CD3^+CD4^+CCR5^+$ T cells co-cultured with ECT1 cells

Because $CD3^+CD4^+$ T cells remain constant in each culturing condition, scrutiny falls to the HIV-1 co-receptors. Further classifying $CD3^+CD4^+$ T cells into $CD3^+CD4^+CCR5^+$ T cells and measuring relative abundance of $CD3^+CD4^+CCR5^+$ T cells out of the parent $CD3^+CD4^+$ T cells provided key insight on how semen modulated immune cells in the subepithelium. Surface expression of CCR5 plays a role in HIV-1 infection and was also evaluated for potential differences due to semen.

$CD3^+CD4^+CCR5^+$ T cells were only 1.85% of the total $CD3^+CD4^+$ T cell population in input cells (Fig. 4.3B). This population reduced 25.4-fold when $CD3^+CD4^+CCR5^+$ T cells were co-cultured with ECT1 cells (Fig. 4.3B). Co-culture of $CD3^+CD4^+CCR5^+$ T cells with the ECT1 epithelium significantly reduced their abundance by 1.78% (Fig. 4.3B). However, when $CD3^+CD4^+CCR5^+$ T cells co-cultured with ECT1 cells were exposed to semen, the percent of $CD3^+CD4^+$ T cells that were positive for CCR5 increased 13.7-fold (Fig. 4.3B). Indeed, analysis via histogram showed a dramatic decrease in the $CD3^+CD4^+CCR5^+$ T cell population when co-cultured with ECT1 cells versus input cells (Fig. 4.3C). This reduction was seen to be partially abrogated by the addition of semen (Fig. 4.3C).

Figure 4.3A

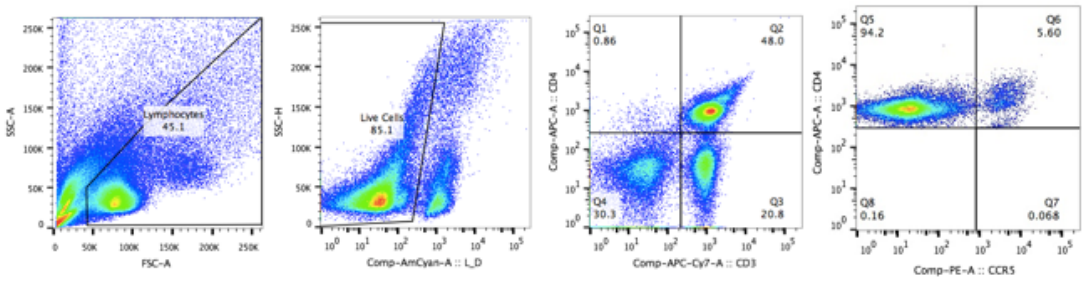

SSC-A
FSC-A
Lymphocytes
45.1
SSC-H
Comp-AmCyan-A :: L_D
Live Cells
85.1
Comp-APC-A :: CD4
Comp-APC-Cy7-A :: CD3
Q1
0.86
Q2
48.0
Q4
30.3
Q3
20.8
Comp-PE-A :: CCR5
Q5
94.2
Q6
5.60
Q8
0.16
Q7
0.068

Figure 4.3B

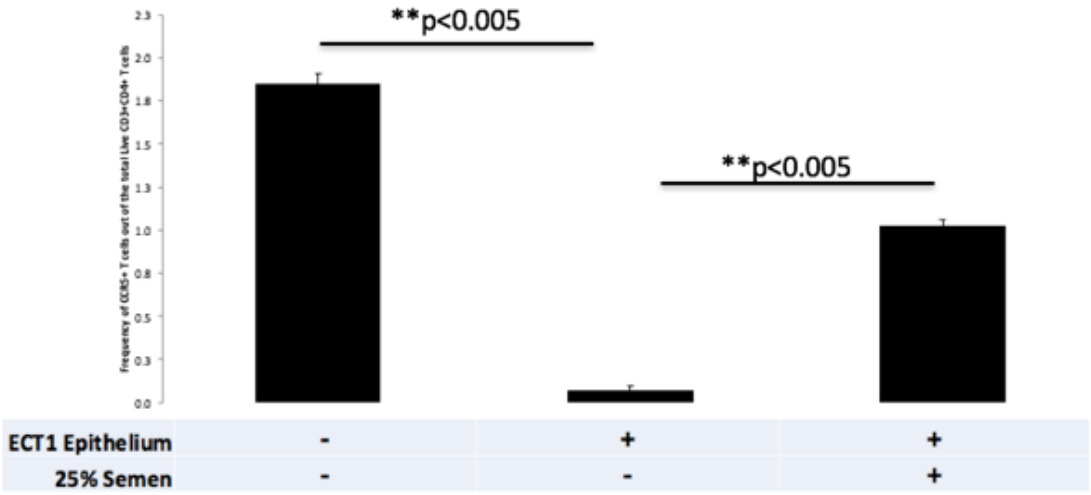

**p<0.005
**p<0.005
Frequency of CCR5+ T cells out of the total Live CD3+CD4+ T cells
2.3
2.0
1.8
1.5
1.3
1.0
0.8
0.5
0.3
0.0
ECT1 Epithelium - + +
25% Semen - - +

Figure 4.3C

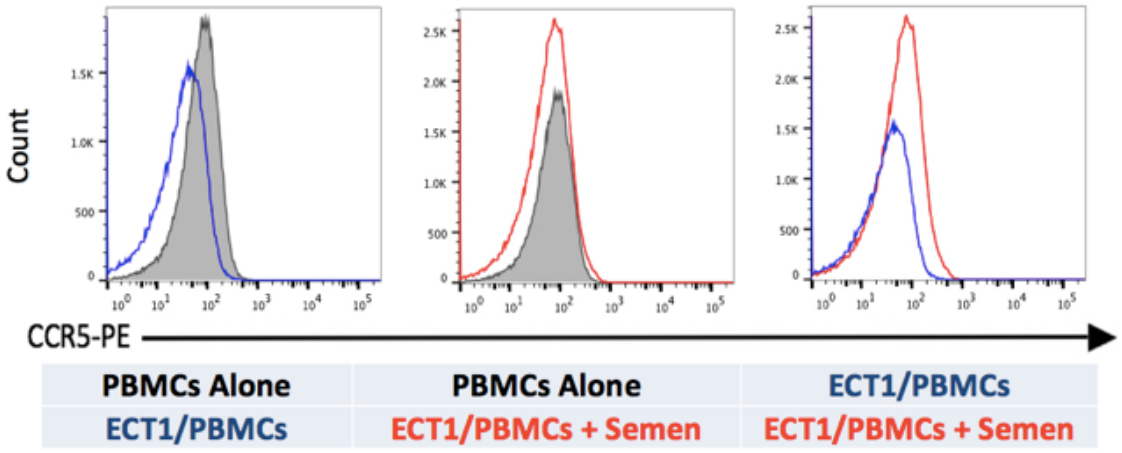

Count
CCR5-PE
PBMCs Alone
ECT1/PBMCs
PBMCs Alone
ECT1/PBMCs + Semen
ECT1/PBMCs
ECT1/PBMCs + Semen

Figure 4.3D

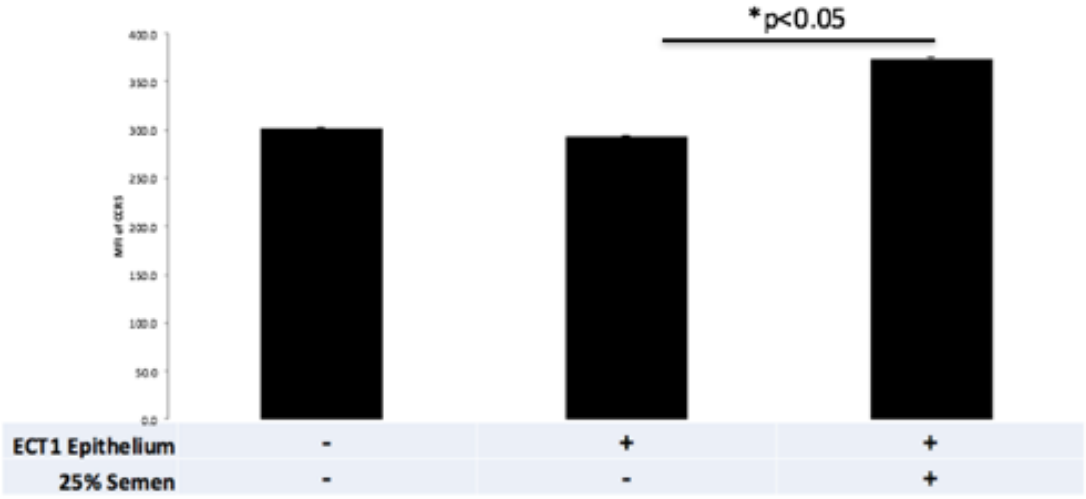

*p<0.05
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
MFI of CCR5
ECT1 Epithelium - + +
25% Semen - - +

Fig. 4.3. Semen prevents the reduction of $CD3^+CD4^+CCR5^+$ T cells.

(**A**) PBMCs were collected from the basolateral chamber of transwells at 4 h post 25% semen exposure from mock and semen wells. The number of $CD3^+CD4^+CCR5^+$ T cells is expressed as a percentage of the total $CD3^+CD4^+$ T cells population. (**B**) Co-culturing of $CD3^+CD4^+CCR5^+$ T cells with the ECT1 epithelium reduced the population of $CD3^+CD4^+CCR5^+$ T cells. (**C**) and (**D**) The surface expression density of CCR5 increased when in the presence of the semen. * is $p<0.05$; ** is $p<0.005$; MFI is median fluorescence intensity; blue is mock treated and red is semen treated.

Interestingly, the cell surface expression of CCR5 on $CD3^+CD4^+CCR5^+$ T cells co-cultured with ECT1 cells was significantly higher when the cells were exposed to semen (Fig. 4.3D). Therefore, despite the severe reduction in population percentages, $CD3^+CD4^+CCR5^+$ T cells co-cultured with the ECT1 epithelium had more CCR5 receptors abundant (Fig. 4.3D) and increased in population (Fig. 4.5B). Taken together, semen elicits a protective effect on $CD3^+CD4^+CCR5^+$ T cells and increases the surface density of CCR5 on said cells.

4.4.6 Surface expression of CXCR4 on $CD3^+CD4^+CXCR4^+$ T cells co-cultured with ECT1 cells is reduced

The next set of experiments explored if semen modulated CXCR4 on $CD3^+CD4^+CXCR4^+$ T cells co-cultured with ECT1 cells. These data, when conferred with the CCR5 data, provided insight into how, and if, semen differentially altered surface expression of HIV-1 co-receptors.

$CD3^+CD4^+CXCR4^+$ T cells comprised 87.5% of $CD3^+CD4^+$ T cells in input cells and when co-cultured with the ECT1 epithelium the population of $CD3^+CD4^+CXCR4^+$ T cells out of the total $CD3^+CD4^+$ T cells did not change, even in the presence of semen (Figs. 4.4B and 4.4C). In fact, the only change in CXCR4 that was evident was surface expression (Fig. 4.4D). The surface expression of CXCR4 on $CD3^+CD4^+CXCR4^+$ T cells from input cells was significantly higher than when $CD3^+CD4^+CXCR4^+$ T cells are co-cultured with the ECT1 epithelium, with or without semen (Fig. 4.4D). Therefore, semen did not enhance, or reduce, CXCR4 surface expression.

Figure 4.4B

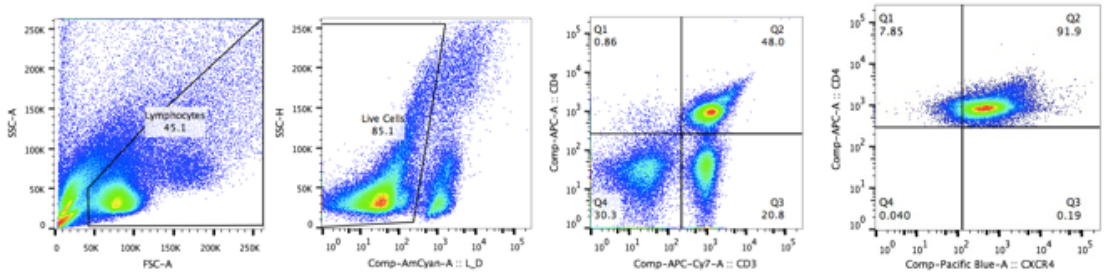

SSC-A
FSC-A
Lymphocytes
45.1
SSC-H
Comp-AmCyan-A :: L_D
Live Cells
85.1
Comp-APC-A :: CD4
Comp-APC-Cy7-A :: CD3
Q1
0.86
Q2
48.0
Q4
30.3
Q3
20.8
Comp-Pacific Blue-A :: CXCR4
Q1
7.85
Q2
91.9
Q4
0.040
Q3
0.19

Figure 4.4C

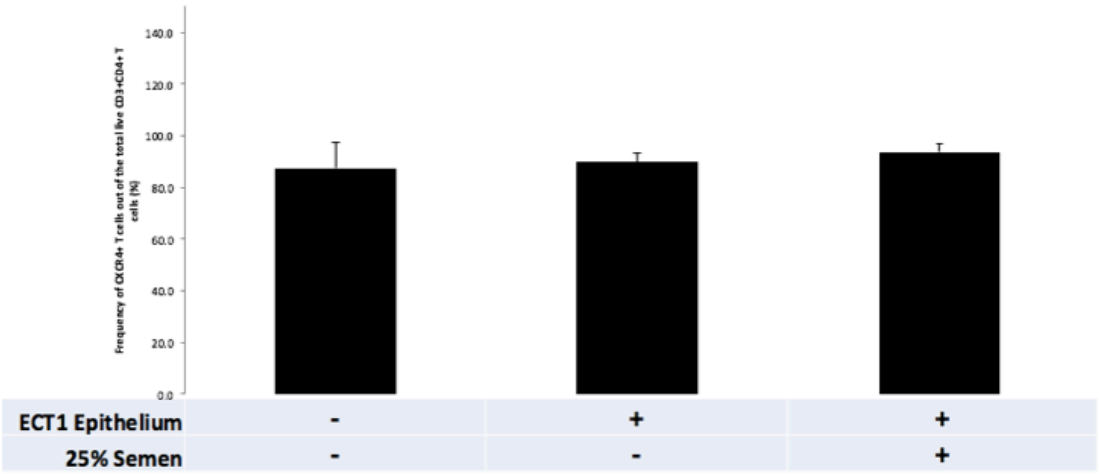

Frequency of CXCR4+ T cells out of the total live CD3+CD4+ T cells (%)
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
ECT1 Epithelium - + +
25% Semen - - +

Figure 4.4C

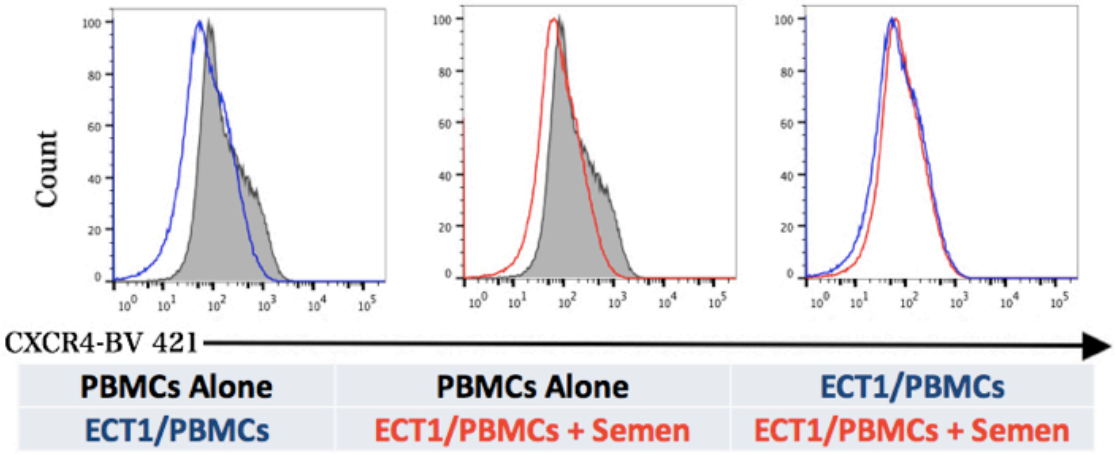

Count
CXCR4-BV 421
PBMCs Alone
ECT1/PBMCs
PBMCs Alone
ECT1/PBMCs + Semen
ECT1/PBMCs
ECT1/PBMCs + Semen

Figure 4.4D

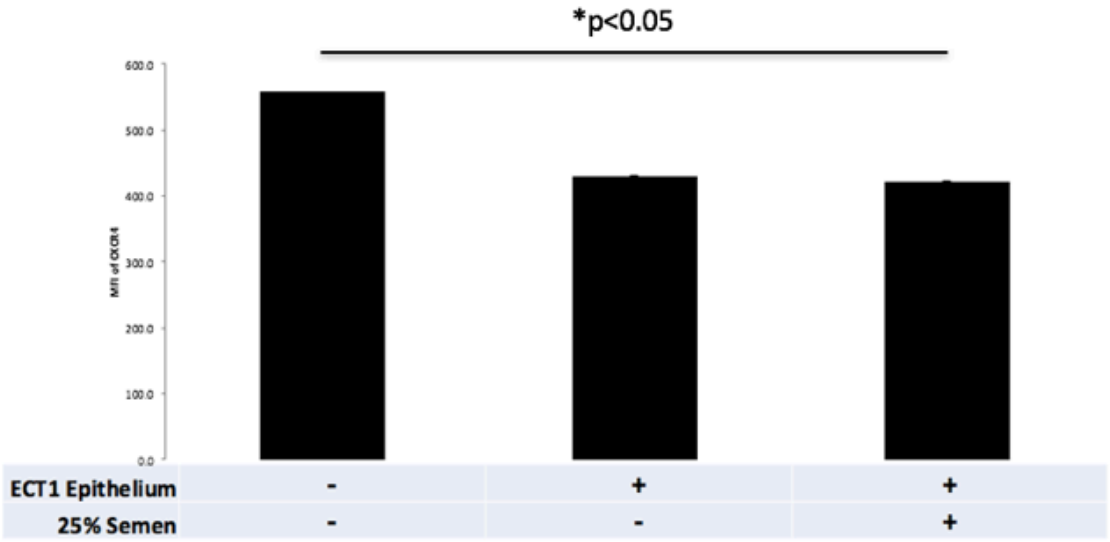

*p<0.05
600.0
500.0
400.0
300.0
200.0
100.0
0.0
ECT1 Epithelium - + +
25% Semen - - +

Fig. 4.4. $CD3^+CD4^+CXCR4^+$ T cells remain unchanged in the presence of semen.

(**A**) PBMCs were collected from the basolateral chamber of transwells at 4 h post 25% semen exposure from mock and semen wells. The number of $CD3^+CD4^+CXCR4^+$ T cells is expressed as a percentage of the total $CD3^+CD4^+$ T cells population. (**B**) Co-culturing of $CXCR4^+$ T cells with the ECT1 epithelium, with or without 25% semen, did not reduce the population of $CD3^+CD4^+CXCR4^+$ T cells. (**C**) and (**D**) The surface expression density of CXCR4 decreases when in the presence of the epithelium, by itself, and when 25% semen is applied to the system. * is $p<0.05$; MFI median fluorescence intensity; blue is mock treated and red is semen treated.

4.4.7 Semen increased infections by R5-tropic viruses by 24.5% when $CD3^+CD4^+$ T cells were co-cultured with ECT1 cells

Our original purpose of elucidating how semen modulates the immune response by ECT1 cells co-cultured with $CD3^+CD4^+$ T cells and what downstream, lasting effects this may have on HIV-1 co-receptors led to identification that semen had a protective effect on $CD3^+CD4^+CCR5^+$ T cells. This effect was not evident with $CD3^+CD4^+CXCR4^+$ T cells and, in fact, semen did not appear to alter populations of $CD3^+CD4^+CXCR4^+$ T cells nor surface expression of CXCR4.

These data suggest semen has an indirect effect on CCR5 and not CXCR4. In order to elucidate if this effect translates to an increase in R5-tropic infection of $CD3^+CD4^+$ T cells an infection assay was performed. Peripheral blood cells co-cultured with ECT1 cells were subject to semen exposure and then immediately collected and challenged with an HIV-1-tropic specific pseudovirus. The pseuodvirus used in this study was created using the HIV-1 genome, except a reporter enhanced green fluorescent protein (eGFP) gene was cloned in the middle of the envelope gene. Because of this, when the virus entered and infected a $CD3^+CD4^+$ T cells it couldn't have undergone a second round of infection due to the fact that an envelope protein was not able to be made and thus prevented the viruses from re-packaging and leaving the cell. Instead of an envelope, eGFP was created and was used as a marker for infection. Therefore, all $CD3^+CD4^+eGFP^+$ T cells are cells that were infected. These data elucidated if semen exposure to $CD3^+CD4^+$ T cells co-cultured with ECT1 cells skewed infection in favor of one HIV-1 tropic strain over the other.

In the absence of semen 13.7% of $CD3^+CD4^+$ T cells were infected by R5-tropic viruses and 26.1% of $CD3^+CD4^+$ T cells were infected by X4-tropic viruses (Fig. 4.5A). However, in the presence of semen 38.2% and 32.6% of $CD3^+CD4^+$ T cells were infected by R5- and X4-tropic viruses, respectively (Fig. 4.5A). Semen enhanced R5-tropic infections by 24.5% and X4-tropic infections by 6.5% (Fig. 4.5A).

Furthermore, the level of eGFP expression correlated to long terminal repeat activity, which, by extension, is a measure of viral gene expression. Therefore, in the absence of semen $CD3^+CD4^+$ T cells infected with X4-tropic virus had 23.8% more viral gene expression than $CD3^+CD4^+$ T cells infected with R5-tropic viruses. However, in the presence of semen viral gene expression was enhanced in $CD3^+CD4^+$ T cells infected by R5-tropic viruses and decreased in $CD3^+CD4^+$ T cells infected by X4-tropic viruses (Fig. 4.5B). Indeed, semen enhanced viral gene expression by 9.56% in $CD3^+CD4^+$ T cells infected by R5-tropic viruses (Fig. 4.5B).

When $CD3^+CD4^+$ T cells were co-cultured with ECT1 cells and exposed to semen, R5- and X4-tropic infections were increased. Semen elicited an effect through the ECT1 epithelium that modulated infection in the basolateral chamber. Semen also transduced its effect through the epithelium to increase viral gene expression in R5-tropic infections and decrease viral gene expression in X4-tropic infections.

Figure 4.5A

ECT1 Epithelium	+	+	+	+
25% Semen	-	+	-	+
R5 Virus	+	+	-	-
X4 Virus	-	-	+	+

Figure 4.5B

ECT1 Epithelium	+	+	+	+
25% Semen	-	+	-	+
R5 Virus	+	+	-	-
X4 Virus	-	-	+	+

Fig. 4.5. R5-tropic infections are increased in the presence of semen.

(**A**) PBMCs from mock and semen exposed wells were collected 4 h after 25% exposure and immediately challenged with either a R5- or X4-tropic pseudovirus infection for 4 h. The cells were then washed and given 48 h recovery. The number of cells infected is expressed as a percent of total $CD3^{+}CD4^{+}$ T cells. (**B**) Viral gene expression can be expressed as a function of GFP median fluorescence intensity because GFP was in place of an envelope protein. Viral gene expression was increased in R5-tropic viruses when exposed to semen, but decreased in X4-tropic viruses, when exposed to semen. MFI is median fluorescence intensity.

4.5 Discussion

The cytokine data generated here used a transwell culture system to recapitulate early aspects in HIV-1 infection from male-to-female. Apical exposure to semen in this system allowed for the direct manipulation and sampling of both the apical and basolateral chambers. When assayed for cytokine content it was found that semen, alone, elicited a robust response from the ECT1 epithelium. These data are supported by other studies involved in cytokine profiling of the FRT [84] (Appendix Chapter 2). Indeed, release of factors like TNF-α and IL-6 from the epithelium promote a pro-inflammatory environment. Again, the release of these factors falls in line with the inflammation first associated with semen deposition in the FRT [29].

However, in *in vivo* conditions the pro-inflammatory response is not mediated by semen alone, but by the interaction between the exposed epithelium and the immune cells present at the time [102-104]. The ectocervix has the highest concentration of immune cells in the subepithelial space of any other portion of the FRT and therefore, the response of the immune cells will drive inflammation due to semen exposure [102]. In order to take the *in vitro* system one step closer to being more physiological PBMCs were co-cultured in the basolateral chamber to ECT1 epithelia grown on the mesh insert. In this respect, the interplay between peripheral blood cells and the epithelium were elucidated and served as the basis for any effects semen had on the epithelium, when co-cultured with PBMCs. Indeed, when co-cultured with ECT1 cells, in the absence of semen, the immune environment in the basolateral chamber was pro-inflammatory in nature with some

analytes, like IL-8 and MCP-1, reaching concentrations up to 58- and 25-fold higher than when PBMCs were absent. TNF-α was almost 10-fold higher in wells that contained PBMCs versus those that did not have PBCMCs. Therefore, the subepithelial space is in constant flux [102, 103] due to the recruitment of fresh immune cells by chemokines like MDC, MIP-1α, and RANTES, which were all further concentrated in wells that contained PBMCs versus those that did not.

Therefore, apical exposure to semen would give insight to how semen may modulate the immune response that was being produced by the peripheral blood cells at constitutive levels. Interestingly, semen had little effect on the release of factors when semen exposed and non-semen exposed basolateral supernatants were compared. Indeed, cytokines released from immune cells, such as IL-12, had a small fold-change in semen exposed wells from non-semen exposed wells. Cytokines involved in immune cell recruitment, such as IL-8, MCP-1, RANTES, and MIP-1β were all increased due to semen exposure. However, it is important to note that where MCP-1 had a 1.14-fold increase in semen exposed wells versus non-semen exposed wells the non-semen exposed wells saw a 25-fold increase. Therefore, cytokines, like MCP-1, compound on their previous fold-changes to bring about a 26.14-fold change in concentration. However, despite the large increases in concentrations caused by the interaction of PBMCs and the ECT1 cells, semen only increases chemokines associated with recruitment.

The recruitment of peripheral blood cells to the ectocervix, especially $CD4^+$ T cells increases the number of targets for HIV-1 to infect. In this *in vitro* system, though, $CD3^+CD4^+$ T cells were not increased due to semen exposure. Of course,

this system is closed and the recruitment of cells can not be measured because the basolateral chamber does not have the capacity for exposure and recruitment at the same time. $CD3^+CD4^+CXCR4^+$ T cells also remained constant in the presence of semen. Indeed, the only T cell subset that diminished in this study were $CD3^+CD4^+CCR5^+$ T cells.

By co-culturing $CD3^+CD4^+CCR5^+$ T cells with the ECT1 epithelium there was a significant reduction in the number of $CD3^+CD4^+CCR5^+$ T cells out of the total $CD3^+CD4^+$ T cells population. Perhaps the fact that MIP-1α, MIP-1β, and RANTES were all increased to higher levels during the co-culture of $CD3^+CD4^+CCR5^+$ T cells and ECT1 cells could explain the loss in $CD3^+CD4^+CCR5^+$ T cells. MIP-1α, MIP-1β, and RANTES are all ligands of CCR5 [135-138], and being high in concentration they may have preferentially been bound to the receptor. However, the surface expression of CCR5 on $CD3^+CD4^+CCR5^+$ T cells did not decrease in any condition, in fact surface expression increased on $CD3^+CD4^+CCR5^+$ T cells collected from semen exposed wells. The surface expression was significantly higher in this condition than in the others. Previous studies have shown that interferon alpha (IFN-a) was highly upregulated in ECT1 cells due to semen exposure (Appendix Chapter 2). Perhaps the increased levels of IFN-a are the cause for the increase in surface expression of CCR5 [139].

When semen is apically applied to the transwell the collected $CD3^+CD4^+CCR5^+$ T cells comprised a higher percentage of the total $CD3^+CD4^+$ T cell population suggesting that semen elicits a protective effect of

$CD3^+CD4^+CCR5^+$ T cells, while increasing surface expression of CCR5. This effect may be due to a factor released from the epithelium in response to semen exposure.

Co-culturing $CD3^+CD4^+$ T cells with the ECT1 epithelium also had deleterious effects on CXCR4 surface expression. In the absence or presence of semen, surface expression of CXCR4 was significantly lower than $CD3^+CD4^+$ T cells that were not exposed to the epithelium, at all. Despite the surface expression of CXCR4 being slightly lowered when co-cultured with the ECT1 epithelium there were still more $CD3^+CD4^+CXCR4^+$ T cells represented than $CD3^+CD4^+CCR5^+$ T cells in the total $CD3^+CD4^+$ T cell population. $CD3^+CD4^+CXCR4^+$ T cells comprise almost 90% of the $CD3^+CD4^+$ T cell population, compared to the 1% of the population that is $CD3^+CD4^+CCR5^+$. This may be due to the overnight IL-2 stimulation. A recent study determined that CCR5 is slowly increased when cultured with IL-2 versus the quick increase in CXCR4 [108]. CCR5 reached peak levels in a longer time in culture with IL-2, whereas CXCR4 was much earlier [108]. The two co-receptors are largely reciprocal and, generally, mutually exclusive [108]. Therefore, the difference in their representation in the total $CD3^+CD4^+$ T cell population may be due to the fact that they were stimulated with IL-2.

Despite the fact that $CD3^+CD4^+CCR5^+$ T cells are underrepresented, over 38% of the population from semen exposed well was infected with a R5-tropic pseudovirus, which was a 24.5% increase from non-semen exposed wells. $CD3^+CD4^+CXCR4^+$ T cells infected with a X4-tropic pseudovirus were only increased by 6.5%. These results differ from the expectations inherent in the

bottleneck hypothesis, which has, at its core, the exclusive acquisition of a single R5-tropic founder virus [130, 132]. Indeed, we report here that X4-tropic pseuodviruses are able to infect $CD3^+CD4^+$ T cells to a comparable degree to R5-tropic pseudoviruses. However, our model system was designed to assess if apical semen exposure could influence greater infection in peripheral blood cells in the basolateral compartment. Unlike what is seen in *in vivo* conditions, the virus was not mixed with semen and then apically applied [98]. Our system assumes that HIV-1 transverses across the FRT and is located in the basolateral compartment. And assuming that HIV-1 is across the epithelium we wanted to understand if semen could enhance infection by acting on the apical side of the epithelium.

Indeed, these results suggest that semen enhances viral infection of immune cells. Perhaps one reason for this is due to semen-derived enhancer of virus infection (SEVI), which has been shown, *in vitro*, to increase HIV-1 infections [140-143]. However, SEVI is still debated as an effector of viral infection [140]. In studies using primary tissue SEVI was found to act differently than it did in *in vitro* models [140]. In primary tissues SEVI binds HIV-1 and sequesters it from the epithelium, preventing impactful penetration of the tissue [140]. Thus, SEVI restricts HIV-1 infection in primary tissues [140]. However, in culture systems SEVI greatly enhances HIV-1 infections [140, 143]. Again, HIV-1 pseudovirus was not added to the semen before apical exposure, so to what extent SEVI has on the basolateral compartment is unknown here. Perhaps SEVI can modulate $CD4^+$ T cells from the luminal side of an epithelium, but that is just speculation.

Curiously, however, in the presence of semen viral gene expression was enhanced in R5-tropic viruses, suggesting that during initial HIV-1 infection viral fitness is increased. X4-tropic associated viral gene expression was decreased due to semen exposure, therefore semen may inadvertently reduce fitness of X4-tropic viruses [144] due to some released factor from the epithelium or immune cells. In the bottleneck hypothesis infection is started by a R5-tropic virus, however there exists a small subset of infections that are initiated by X4-tropic viruses [130]. Perhaps both virus types are present in the subepithelium, but R5-tropic viruses become enhanced by semen, thus establishing infection quicker than X4-tropic viruses. Indeed, assuming both virus types are present in the subepithelium, this study reports that R5-tropic viruses will more easily enter and infect $CD3^{+}CD4^{+}$ T cells due to semen.

This study took a simplistic approach to the very complex issue. However, the presence of semen on the apical side of an epithelium enhances R5-tropic infections to a greater degree than X4-tropic infections. Semen has even been shown to reduce X4 viral fitness by reducing the viral gene expression. Taken together, semen preferentially enhances R5-tropic infections *in vitro*. The bottleneck hypothesis is a series of checkpoints filtering out viruses until there is only a single isolate that initiates infection in the FRT [130, 132] and this study adds another clue as to why the interaction between the FRT and semen is the most stringent bottleneck. Future studies will need to address the limitations of an *in vitro* system.

Chapter 5

Semen differentially modulates HIV-1 pseudovirus infection in peripheral blood mononuclear cells co-cultured with cervicovaginal epithelial cells from the female reproductive tract

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5.1 Abstract

The female reproductive tract (FRT) is comprised of three distinct anatomical regions. The epithelia in each region – the vagina, ectocervix, and endocervix – all respond to foreign stimuli differently, which may result in regional differences in the risk of pathogen infection. In the context of human immunodeficiency virus type 1 (HIV-1) transmission, epithelial responses to foreign stimuli may alter the susceptibility of subepithelial immune cells to viral infection, particularly with respect to viral co-receptor-tropism. HIV-1 tropism plays a major role in male-to-female HIV-1 transmission; R5 viruses (those that use CD4 and CCR5 for entry) are highly favored during heterosexual transmission over X4 viruses (viruses that use CD4 and CXCR4 for entry). Using three cell lines derived from the three regions of the FRT, we report that the differential response to semen by ECT1 cells favors R5 viral transmission of HIV-1 over X4, where VK2 and END1 cells favor X4 viral transmission of HIV-1. These data provide new insights into the underlying mechanisms of HIV-1 transmission and may point to new opportunities for preventing HIV-1 acquisition in women at risk for infection.

5.2 Introduction

Although the introduction of semen into the female reproductive tract (FRT) is a key step in conception [114], semen is not just an inert vehicle for sperm delivery. Rather, semen is comprised of immunomodulatory factors, whole cells, sugars, and other factors [29]. These constituents modulate responses in the FRT that are critical for successful conception [29]. It is known that semen has a biphasic effect in the FRT, characterized by initial inflammation followed by changes that lead to immunological tolerance [5, 99]. In doing so, semen ensures the survival of sperm cells that would otherwise be targeted by the female immune system [9, 29]. Therefore, semen plays an integral part in not only in conveying sperm cells but also in the manipulation of immunological responses of the FRT [28, 84, 99].

The FRT functions as disparate regions [103, 104] that are separated into different regions: the vagina, the cervix, and the uterus. The vagina is the region closest to the outside of the body and is characterized by a multi-layered, squamous epithelium [104]. The cervix is divided into two separate regions: the ectocervix and the endocervix. The ectocervix is the lower portion of the cervix where the vaginal epithelium meets the cervical epithelium [104]. Both the vagina and the ectocervix have stratified, squamous epithelia that help protect the underlying tissue from pathogens and foreign antigens. In contrast to the vagina and ectocervix, the endocervix (the upper portion of the cervix) is thought of as a sterile environment [103]. Comprised of a single layer of columnar cells, the relatively thin epithelium of the endocervix may be a more likely site for a pathogen

to cross the epithelial barrier. However, the location of the endocervix reduces the probability that these tissues will be exposed to outside stimuli or pathogens [126]. The endocervix is also unique in that it has a layer of cervicovaginal mucus covering the luminal side of the epithelium. This mucus has been shown to “trap” particles and slow their progression of coming in contact with the epithelial barrier, and any immune cells that may be found interstitially in the barrier [145]. The mucus may also have some anti-human immunodeficiency virus type 1 (HIV-1) properties aside from physically slowing down the virus so it becomes sloughed off when the mucus is refreshed in the FRT [145, 146].

Semen, which can also be considered a foreign stimulus, does not come in direct contact with the endocervix [9]. The vagina and, sometimes the ectocervix, are the primary regions that come in physical contact with semen. However, the presence of semen in the other portions of the FRT has consequences for the tract as a whole. Indeed, previous studies that characterized semen’s effect on the FRT have shown that each region acts differently when exposed to semen [29, 84, 95, 99, 100, 103, 104, 106] (Appendix Chapters 1 and 2). When cell lines representing each region of the FRT were apically exposed to semen each cell line had a unique time-, direction-, and tissue-dependent release of immunomodulatory factors (Appendix Chapter 1). ECT1/E6E7 (ECT1) cell lines, representing the ectocervix, showed the largest response to semen exposure. The least responsive cell line was END1/E6E7 (END1), which represents the endocervix (Appendix Chapter 1). The ECT1 cell line having the largest response to foreign stimuli is due to the fact the ectocervix is also the transformation zone [29, 103]. This region is the portion

of the cervix where the squamous, stratified epithelium of the ectocervix transitions into the single columnar epithelium of the endocervix [103]. The transformation zone is viewed as the central hub for cell mediated immunity in the FRT due to the increased presence of subepithelilal antigen presenting cells (APCs), dendritic cells (DCs), monocytes, macrophages, and T lymphocytes as compared to the other regions [95, 102-104]. Indeed, the ectocervix contains a higher concentration of immune cells than the vagina, which, in turn, hosts a larger population of immune cells than the endocervix [102]. This may be due, in part, to the fact that the endocervix is located higher up in the FRT (more distal from the vaginal opening), which, again, makes it more sterile than the other regions that are routinely exposed to external antigens [102]. In fact, the ectocervix is suggested as a "hot-spot" for HIV-1 transmission; however that does not preclude the other regions from supporting transmission [4, 5] (Appendix Chapter 1, Appendix Chapter 2).

Immunomodulation by semen also appears to increase the risk of pathogen transmission [9, 29]. Semen has been shown to increase leukocyte recruitment to the site of deposition, which, again, is part of the biphasic effect of semen [28, 61, 99, 106, 109, 127, 147]. The initial inflammation is replete with an influx of immune cells, many of which are $CD4^+$ T lymphocytes, such as T_h17 cells and T regulatory cells (Tregs) [5, 77, 109, 124]. In the case of human immunodeficiency virus type 1 (HIV-1), the introduction of more T lymphocytes may increase the likelihood of HIV-1 transmission because of the increase in target cells. Furthermore, the resolution of the effect of semen – tolerance – may further increase the risk of HIV-1 transmission [95, 126, 139, 147]. Tolerance in the FRT, due to semen, is

mediated by the recruitment of Tregs [5, 124, 125]. These Tregs will further dampen the immune response and induce a state that may be favorable to HIV-1 transmission [103, 104]. Tregs produce high concentrations of transforming growth factor beta (TGF-ß) and interleukin 10 (IL-10), which will induce naïve T cells to differentiate into more Tregs, further enhancing Treg proliferation (Chapter 3).

Just as important as available immune cells are to male-to-female HIV-1 transmission so is viral tropism. HIV-1 tropism is defined by co-receptor usage during the initial steps of infection. R5 viruses enter cells via interactions with CD4 and the chemokine receptor CCR5, while X4 viruses use CXCR4 as the co-receptor. The bottleneck hypothesis of HIV-1 transmission attempts to explain the phenomenon that R5 viruses are almost exclusively transmitted between partners in heterosexual, discordant couples [130, 132]. During heterosexual transmission of HIV-1, R5 viruses are generally the transmitted/founder virus—the virus that initiates infection by draining to the lymph nodes [132]. That is not to say that X4 viruses never initiate infection, it's just more uncommon, which may be partly explained by the most stringent checkpoint in the bottleneck hypothesis—when semen comes in contact with the FRT [130] (Chapter 4). A recent finding by our group has shown that in the absence of semen X4 viruses were the most common virus to infect $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells (Chapter 4). However, when $CD3^+CD4^+$ T helper cells were co-cultured with ECT1 cells in the presence of semen, the bias toward R5 viruses was seen, which suggested that there was a factor (or more) released from the epithelium in response to semen that aided R5 viruses in entry and infection of target cells (Chapter 4).

The bottleneck hypothesis is not about a singular checkpoint that filters all viruses down to one specific genetic variant, but multiple checkpoints that filter out variants over the course of transmission. Because of this the most stringent bottleneck would be when semen comes in contact with the FRT [130, 132]. Once in contact the checkpoint becomes the female, who has defenses like cervicovaginal mucus. The mucus, for example, contains a myriad of factors, one of them being lectins—which may preferentially bind to heavy methylated HIV-1 virions and, thus, prevent them from being passed on to the female [132, 146]. Therefore, each portion of the FRT could be a different filtering process that might leave one area, or more, as a place where R5 viruses are favored and, thus, promote R5 HIV-1 transmission. From previous work we have demonstrated that each region of the FRT responds differently to semen exposure with respect to immunomodulatory factor release, therefore each region of the FRT might serve as a different checkpoint for filtering out genetic variants (Appendix Chapter 1). The notion of each region acting independently of one another is not new.

Previous studies of the FRT epithelial cells have used submerged cultures, in which representative cell lines are maintained on a flat surface. Unfortunately, this approach is not physiologically relevant since submerged cultures prevent the manipulation and scrutiny of the basolateral side of the cells. To circumvent this limitation, our previous studies used a transwell cell culture system (Appendix Chapter 1, Appendix Chapter 2) that allows for the polarized cell growth and independent manipulation of the apical and basolateral media [8, 148]. Using this transwell culture system, we demonstrated a time-dependent and polarized

release of cytokines from epithelilal cells of the FRT in as little as 4 h post semen exposure (Appendix Chapter 1). Indeed, ECT1 cells had the largest response to semen with cytokines like interleukin 12 (IL-12), interleukin 6 (IL-6), interleukin 7 (IL-7), vascular endothelial growth factor (VEGF), and interleukin 2 receptor (IL-2r) all having concentrations increase greater than 10-fold change over non-semen exposed monolayers (Appendix Chapter 1). The next largest response came from VK2 cells exposed to semen, which displayed fewer cytokines that had a greater than 10-fold change over non-semen exposed monolayers (only IL-7 and interleukin 15 (IL-15)) (Appendix Chapter 1). The least responsive were END1 cells, which had no cytokines reach a 10-fold change over non-semen exposed monolayers (Appendix Chapter 1).

Identifying semen's interaction with each cell line was only a starting point. Here we take the polarized FRT *in vitro* model system further by incorporating peripheral blood mononuclear cells (PBMCs) to assess how the interplay between epithelial cells and semen acting on the immune cells may modulate the immune environment in the subepithelial space. Once it was identified how semen changed the immune environment the next step was to elucidate if this effect by semen further modulated the PBMCs co-cultured with each cell line to either be more or less susceptible to infection by R5 or X4 HIV-1 pseudovirus. Our findings suggest that the pro-inflammatory environment induced by semen exposure had an effect that increased infection of X4 pseuodviruses in PBMCs co-cultured with each cell type. However, semen exposure only increased R5 infections in $CD3^{+}CD4^{+}$ T helper cells co-cultured with ECT1 cells, while decreasing R5 infections in

$CD3^+CD4^+$ T helper cells cultured with VK2 or END1 cell lines. Therefore, semen transduces its effect through each epithelium to produce disparate outcomes in each cell line, thus in each region of the FRT. These effects may contribute to establishing preferred sites of HIV-1 transmission and to the apparent transmission bottleneck.

5.3 Materials and Methods

5.3.1 Cell line maintenance

Experiments were performed using cell lines derived from three regions of the human female reproductive tract: vaginal keratinocytes (VK2/E6E7), ectocervical cells (ECT1/E6E7), and endocervical cells (END1/E6E7) [133] . Each cell line was retained in T150 tissue culture flasks (Grenier Bio One, Monroe, NC) at 37°C with 5% CO_2. All cell lines were cultured using Keratinocyte Serum-Free Medium (Gibco, Grand Island, NY), with the added supplements of 50 µg/ml bovine pituitary extract (BPE), 0.1 ng/ml epithelial growth factor (EGF), 50X penicillin/streptomycin solution, and 0.4 mM calcium chloride (EMD Millipore, Billerica, MA).

5.3.2 Transwell culture system

Confluent, polarized monolayers of each cell line were cultured by seeding into the apical chamber of a 6.5 mm, 0.4 µm pore size, polyester transwell insert in a 24-well receiver plate (Corning, Corning, NY). Cells were seeded in the apical chamber at a density of 1.5×10^5 cells in 200 µl. Media, in a volume of 600 µl, was

aliquoted in the basolateral chamber. Time to confluence varied between 14 and 17 days post-seeding and was measured by transepithelial electrical resistance (TEER). TEER readings were taken every other day to moniter the growth and potential use of the transwell plate.

5.3.3 Semen

Semen from more than 3 healthy donors between the ages of 26 and 34 years was pooled and purchased in 20 ml volumes (Lee Biosolutions, St. Louis, MO). Samples were shipped overnight on ice and immediately separated into 500 µl aliquots and frozen at -20°C upon arrival. In all descriptions, semen refers to whole, unfractionated semen.

5.3.4 Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were obtained from the Comprehensive NeuroAIDS Center (CNAC) Mammalian Cell and Virus Core cohort at Temple University. PBMCs were then cultured overnight in RPM1-1640 supplemented with 2% (10 ml) heat-inactivated FBS and 1% (5 ml) HEPES (R1H medium). Interleukin 2 (10 U/ml) was added to the medium for overnight activation and to ensure T lymphocyte survival. After 16-18 h incubation the cells were then spun down at 2500 rpm for 5 minutes. Cells were then resuspended in the appropriate amount of R1H to reach a concentration of 1×10^6 cells/ml. Unless stated otherwise, PBMCs were seeded at 1×10^6 cells/ml.

5.3.5 Collection of conditioned media

Transwells containing confluent cell line monolayers were washed twice with HBSS. For some wells, the basolateral chamber was then supplemented with

600 µl of 1 million IL-2-activated PBMCs. For other wells, PBMCs were not added. The apical chambers were then supplemented with 25% semen in duplicate for 4 h for all wells, in the absence or presence of PBMCs. After 4 h, basolateral media containing PBMCs were collected and spun down at 2500 rpm for 5 minutes. The supernatant was collected and stored at -80°C. For wells not containing PBMCs, the supernatants was collected immediately after 4 h apical exposure to 25% semen. The PBMCs were kept for further analyses (see below). Wells with just the cell line monolayers and without 25% semen or PBMCs were designated mock wells, while wells with the cell monolayers and PBMCs, but without 25% semen were termed PBMCs. Wells with the cell line monolayers, PBMCs, and 25% semen were designated semen.

5.3.6 ELISArray

The collected supernatants were analyzed by the Qiagen Human TLR-induced Cytokines II: Microbial-induced Multi-Analyte ELISArray assay (Qiagen, Maryland). Conditioned media samples were analyzed as directed by the manufacturer's instructions. The results were analyzed in Microsoft Excel.

5.3.7 Flow Cytometry

PBMCs collected from each transwell exposed apically to 25% semen were pelleted to separate cells from conditioned media. The cells were then resuspended in R1H medium for antibody staining and flow cytometry. Anti-CCR5 antibody conjugated to PE (5 µl) (eBioscience, San Diego, CA) was applied first and alone. The antibody was left to incubate for 45 minutes at room temperature (25 ºC). The cells were then washed with 2 ml of R1H and pelleted at 2500 rpm for

5 minutes. The PBMCs were then resuspended in a volume of R1H medium for further staining. Anti-CD4 antibody (5 µl) conjugated to APC (eBioscience, San Diego, CA), 5 µl of anti-CXCR4 antibody conjugated to BV 421 (BioLegend, San Diego, CA), and 3 µl of anti-CD3 antibody conjugated to APC-Cy7 (BioLegend, San Diego, CA) were then added to each tube. The antibodies were left to incubate for 40 minutes at 4ºC. After 40 minutes, 1 µl of a Live/Dead stain, conjugated to BV 510 (AmCyan or Aqua) (eBioscience, San Diego, CA) was added to each tube. The final solution was incubated at 4 ºC for another 15 minutes. After the final incubation, the PBMCs were washed with 2 ml R1H medium and pelleted at 2500 rpm for 5 minutes. The cells were then resuspended in 500 µl of 1% paraformaldehyde (PFA) for 40 minutes at room temp. After incubation, the cells were washed with 2 ml R1H medium, pelleted at 2500 rpm for 5 minutes, and then resuspended in 500 µl of a filter-sterilized buffer consisting of 500 ml of HBSS supplemented with 3% (15 ml) heat-inactivated FBS, 2.5 mM (1.25 ml of a 1 M solution) of $CaCl_2$, and 0.02% (0.1 g) sodium azide (Flow/Facs Buffer). The cells were then analyzed via flow cytometry using an LSR Fortessa. All data were analyzed in FlowJo software (version X.0.7) and Microsoft Excel.

5.3.8 Pseudovirus Propagation

Pseudoviruses were created using the HIV-1 pseudovirus plasmid pNL4.3-∆E-eGFP (NIH AIDS Reagent Program, Bethesda, MD) and expression vectors containing gp120 coding sequences from HIV-1 strain HXB2 and a previously identified transmitted founder (T/F) strain (NIH AIDS Reagent Program Reagent p1012.TC21.3257). The HIV-1 pseudovirus plasmid and one envelope plasmid

were co-transfected into HEK 293 cells (ATCC, Manassas, VA). Media samples containing nascent pseudoviruses were collected 48 h post-transfection and stored at -80 ºC until needed. The substitution of the envelope gene for an eGFP gene ensures that the viral particles are transcribed and translated but not packaged so the virus cannot leave and re-infect another cell. In lieu of the envelope eGFP protein will be made and can be used as a marker for infection

5.3.9 Pseudoviral Infections

Pseudoviruses with either HXB2 (X4) or T/F (R5) envelopes were used in transmission studies. Immediately after a 4 h 25% semen exposure, each transwell insert was removed. In the exposed basolateral chamber containing PBMCs, as previously described, 600 µl of diluted pseudovirus of one envelope type was added. X4 pseudovirus was diluted down 1:10 and R5 pseudovirus was diluted down to 1:4 to ensure even GFP and infection signal. After a 4 h infection, the entire volume of media in the basolateral chamber was collected and centrifuged at 2500 rpm for 5 minutes. With the supernatant discarded, the PBMCs were then resuspended in R1H medium for staining. Anti-CD4 antibody (5 µl) conjugated to APC and 3 µl of anti-CD3 antibody conjugated to APC-Cy7 were added to each tube. After incubating for 40 minutes, a Live/Dead stain conjugated to BV 521 was added and the tubes incubated for another 15 minutes. The cells were then washed with 2 ml R1H medium and pelleted at 2500 rpm for 5 minutes. The PBMCs were then resuspended in 500 µl of 1% PFA for 40 minutes. After incubation, the cells were washed, pelleted, resuspended in 500 µl of Flow/Facs

Buffer, and immediately assayed on a LSR Fortessa flow cytometer. All data were analyzed in FlowJo software (version 10.1r5) and Microsoft Excel.

5.3.10 Statistical analyses

p-Values were calculated for the difference between pairs of treatments conditions using a two-tailed Student's *t*-test, and a value of less than 0.05 was set as the limit for statistical significance. p-Values are indicated on the figures and within the figure legends.

5.4 Results

5.4.1 END1 cells were the most responsive to semen

When semen comes in contact with each region of the FRT, it transduces its effect to the underlying tissue and creates a specific immune microenvironment [84, 103]. Therefore, our first objective was to assess the effect of semen on basolateral cytokine release and to compare cytokine release profiles from all three regional cell lines. When three different cell lines representing the distinct regions of the FRT were cultured on transwells and either exposed to media only (referred to as mock) or semen, distinct patterns of cytokine release from each cell line were observed (Fig. 5.1A). Across each region, semen induces microenvironments that are pro-inflammatory in nature [103]. In these experiments, interleukin 8 (IL-8) increased in concentration approximately 2-fold, 3-fold, and 3.4-fold over mock in the VK2, ECT1, and END1 cell line, respectively (Fig. 5.1A). In fact, IL-8 was held constant as the cytokine that had the greatest fold change over mock in all three regions of the FRT (Fig. 5.1A).

Most of the other analytes appeared to differ in fold-change over mock in each region of the FRT, with no two regions having the same response (Fig. 5.1A). IL-12, for example, had about a 0.7-fold change increase over mock in VK2 cells, about a 1.0-fold change over mock in ECT1 cells, and a moderate ~0.9-fold change over mock in END1 cells (Fig. 5.1A). Regulated on activation, normal T cell expressed and secreted (RANTES) had about a 1.0-fold change over mock in VK2 cells and ECT1 cells, but going up to ~2.0 fold-change over mock in END1 cells, highlighting regional differences (Fig. 5.1A). Previous studies have pointed to ECT1 cells as being the most responsive to semen (Appendix Chapter 1). In these experiments, however, END1 cells had the largest response to semen. Cytokines like interleukin 6 (IL-6), interleukin 17A (IL-17A), RANTES, and MDC all had about a 1.9-fold change over mock in END1 cells, where in the other regions they were either ~1.0-fold change over mock or less (Fig. 5.1A).

5.4.2 ECT1 cells were the most responsive when co-cultured with PBMCs in the basolateral chamber

As the final goal was to examine the effect of semen on an early infection model that included polarized FRT epithelial cells as well as subepithelial immune cells, experiments were performed to examine the impact of adding PBMCs to the basolateral chamber of the transwell model. Since peripheral immune cells release their own immunomodulatory factors and presumably respond to factors released constitutively by the epithelial cells, these experiments were a necessary to establish baselines for experiments also involving semen.

Figure 5.1A

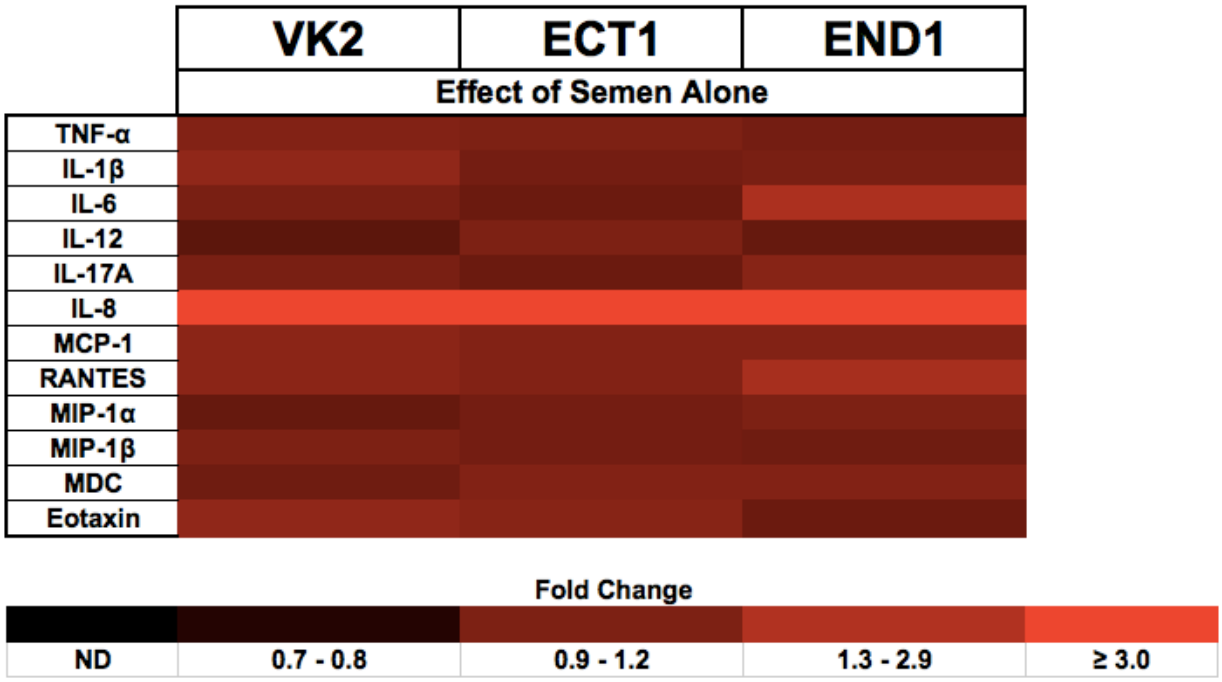

VK2
ECT1
END1
Effect of Semen Alone
TNF-α
IL-1β
IL-6
IL-12
IL-17A
IL-8
MCP-1
RANTES
MIP-1α
MIP-1β
MDC
Eotaxin
Fold Change
ND
0.7 - 0.8
0.9 - 1.2
1.3 - 2.9
≥ 3.0

Figure 5.1B

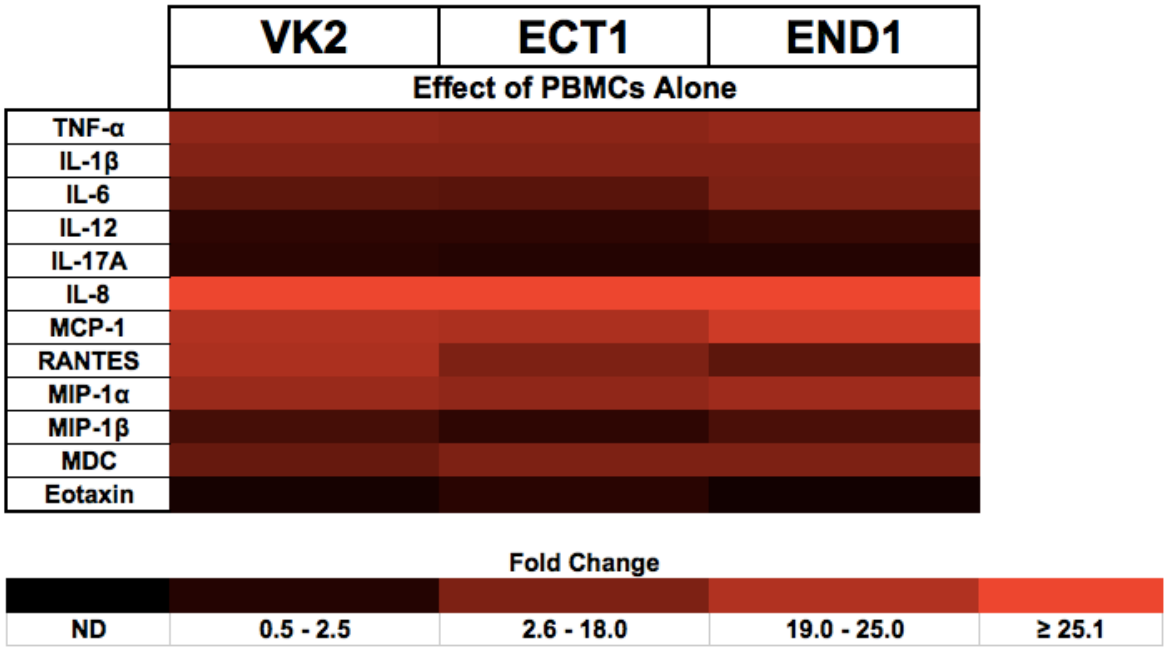

VK2
ECT1
END1
Effect of PBMCs Alone
TNF-α
IL-1β
IL-6
IL-12
IL-17A
IL-8
MCP-1
RANTES
MIP-1α
MIP-1β
MDC
Eotaxin
Fold Change
ND
0.5 - 2.5
2.6 - 18.0
19.0 - 25.0
≥ 25.1

Figure 5.1C

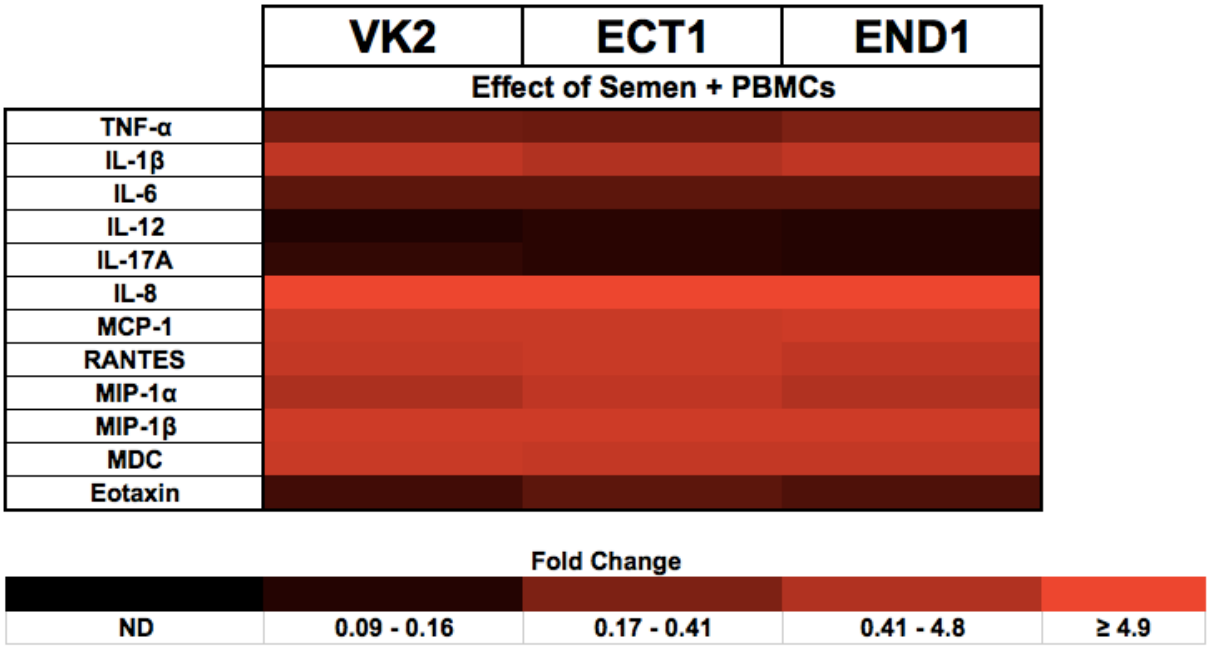


Fig. 5.1. Pro-inflammatory cytokines released basolaterally in response to semen and PBMCs treatment in each cell line. Changes in factor concentrations in the basolateral chambers are shown as a heat map of fold-change over mock of 12 analytes detected in conditioned media collected from the basolateral supernatants at 4 h after epithelial exposure to either **(A)** 25% semen, **(B)** PBMCs, or **(C)** both together.

Each cell line was grown to a confluent monolayer on an insert of a transwell. Once confluency was reached, as measured by TEER, PBMCs, grown overnight in interleukin 2 (IL-2), were co-cultured in the basolateral chamber for 4 h. After exposure the basolateral chamber was collected and the supernatant was assayed for cytokine concentrations, which was expressed as a fold-change over transwells that were not exposed to PBMCs (control). The concentrations of cytokines released by each cell line were relatively similar with some exceptions (Fig. 5.1B). Indeed, an increased concentration (greater than ~25-fold change over control wells) in IL-8 and almost a 1.0-fold change in concentration of IL-12, IL-17A, macrophage inflammatory protein beta (MIP-1ß), and Eotaxin was seen in all regions (Fig. 5.1B). Most notable were the region specific differences, such as RANTES going from ~14.5-fold change over control wells in VK2 cells to just above 5.0-fold change over control wells in ECT1 cells to half that (~2.5-fold change over control wells) in END1 cells (Fig. 5.1B). Aside from RANTES, ECT1 cells had the highest fold-changes of cytokine concentrations over control wells than the other cell lines (Fig. 5.1B). Tumor necrosis factor alpha (TNF-α) had the highest fold-change over control wells in ECT1 cells (~9.5-fold change) when compared to VK2 and END1 cells (both ~7.5-fold change) (Fig. 5.1B). Almost any other analyte had the highest fold-change over control wells in ECT1 cells when compared to the other cell lines, suggesting that ECT1 cells had the largest response to immune cells at basal level (Fig. 5.1B).

5.4.3 Semen did not profoundly affect cytokine concentrations released by FRT regional cell lines co-cultured with PBMCs

A baseline for cytokine concentration during co-culture of each cell line and PBMCs had been established. The final step in assaying semen's effect on an early infection model was to expose polarized FRT epithelial cell lines co-cultured with PBMCs to semen. Analysis of the supernatant from each basolateral chamber 4 h post exposure gave insight to the response of each cell line. Cytokine concentration was expressed as a fold-change over wells that were co-cultured but without exposure to semen (termed non-semen exposed wells).

Cytokine concentrations of most analytes were similar for each cell line (Fig 1C). As with the other conditions, IL-8 had the largest increase over non-semen exposed wells (all above ~4.9-fold change) (Fig. 5.1C). In fact, cytokines that stimulate growth and differentiation were increased less over non-semen exposed wells relative to factors responsible for immune cell recruitment, except for IL-8 (Fig. 5.1C). Cytokine concentrations for MCP-1, RANTES, and MDC were all around a 1-fold change over non-semen exposed wells in each cell line (Fig. 5.1C). Macrophage inflammatory protein one alpha (MIP-1α) and IL-1ß has similar concentrations of about 0.5-fold change over non-semen exposed wells in all three cell lines (Fig. 5.1C). The rest of the analytes had concentrations below a ~0.4-fold change over non-semen exposed wells in each cell line (Fig. 5.1C). Each cell line had analyte concentrations that were very similar to one another as well as what was observed after PBMC-only expsoure (Fig. 5.1B and 1C). The effect of semen on each cell line co-cultured with PBMCs may have been too small, compared to

the effect of each cell line co-cultured with just PBMCs, alone, to have been evident. That is not to say that semen's effect was not seen, it was just that the immune cells further enhanced the effect of semen. Indeed, cytokine concentrations from cell lines co-cultured with PBMCs and exposed to semen followed the same pattern as that of PBMCs alone; ECT1 cells were the most responsive, and had the greatest fold-change over non-semen exposed wells of the analytes (Fig. 5.1C). Compared to the other cell lines ECT1 cells, as before, had either comparable cytokine concentrations or the greatest concentrations.

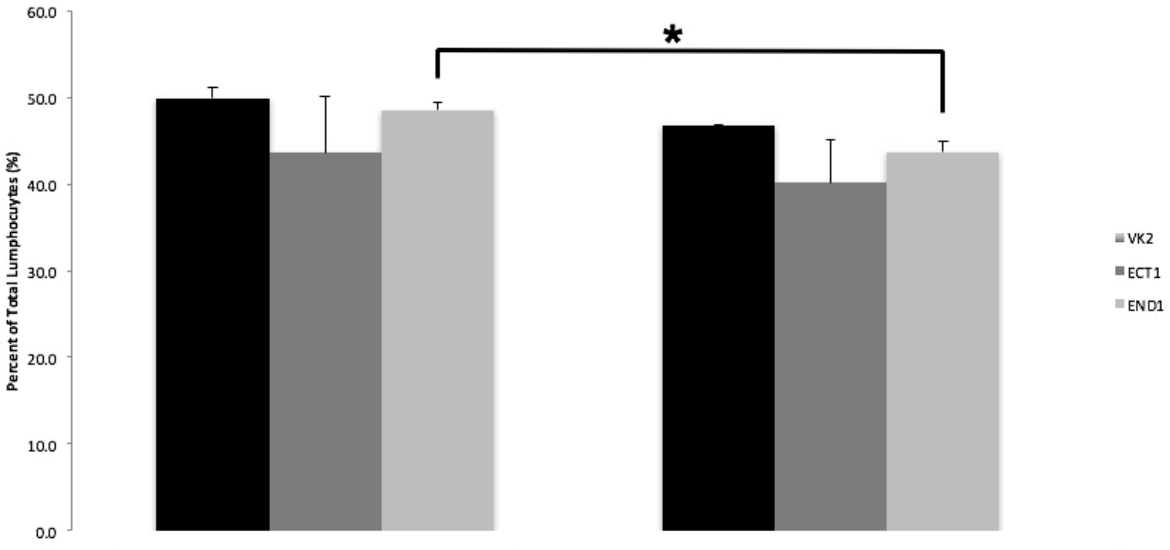
5.4.4 Semen decreased the surface expression of CD4 on $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells but not when co-cultured with VK2 or END1 cells

Once the effect semen had on immunomodulatory factors released from each cell line was measured the next step was to identify how that effect modulated the co-cultured PBMCs and their potential susceptibility to HIV-1 infection. PBMCs from the experiments above were isolated and evaluated for the abundance of HIV-1 target cells, $CD3^+CD4^+$ T cells (T helper cells), and the cell surface expression of CD4. These experiments were performed to test the hypothesis that regional differences in the response of each cell line to semen exposure result in differences in the availability of HIV-1-susceptible T lymphocytes and their ability to support infection by R5 or X4 viruses.

With respect to T helper cells, there was not much of a difference between non-semen exposed wells or semen exposed wells (Fig. 5.2A). Indeed, the percent

of T- lymphocytes that were $CD3^{+}CD4^{+}$ did not change when VK2 wells or ECT1 wells were exposed to semen (Fig. 5.2A). When non-semen exposed wells and semen exposed END1 wells were compared with themselves, however, there appeared to be a significant (~5%) reduction in T helper cells (Fig. 5.2A). Despite the reduction in T helper cells in END1 wells, the surface expression of CD4 on T helper cells co-cultured with END1 cells was significantly enhanced in the presence of semen when compared to T helper cells co-cultured with ECT1 cells by about 37.5% (Fig. 5.2B). This effect was not observed when comparing END1 and VK2 cells (Fig. 5.2B). When compared the other cell lines, T helper cells co-cultured with ECT1 cells had the significantly lowest surface density of CD4 in the absence or presence of semen as compared to the other cell lines by about 30.3% and 37.5% for VK2 and END1 cells, respectively (Fig. 5.2B).

Figure 5.2A



Epithelium	+	+
25% Semen	-	+

Figure 5.2B

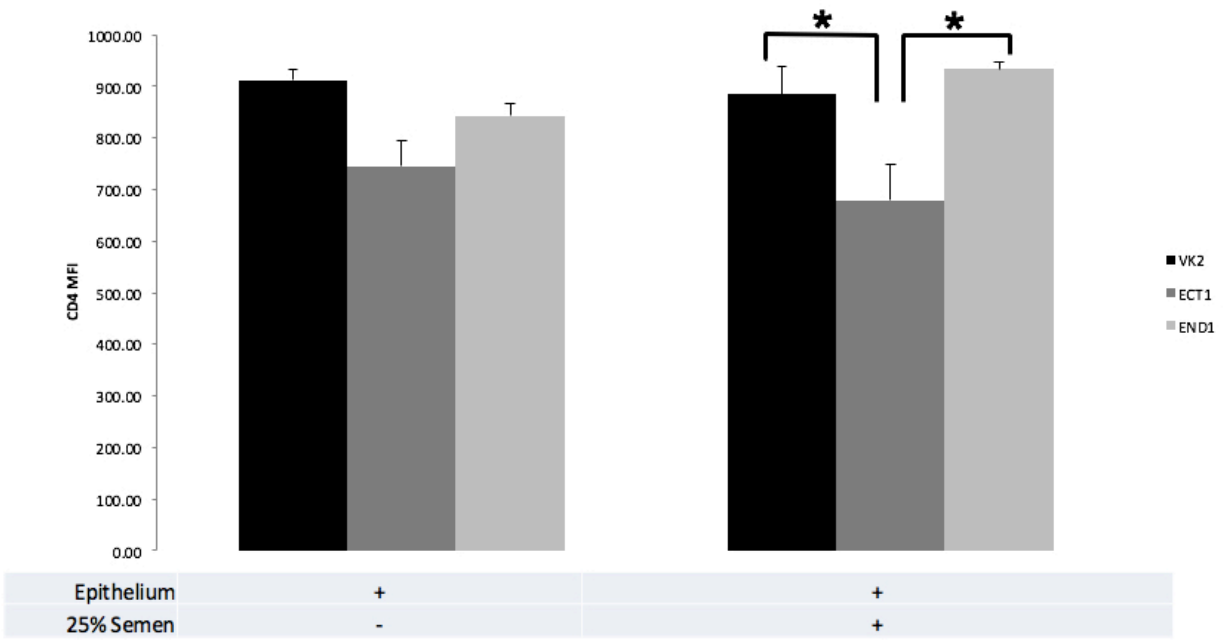


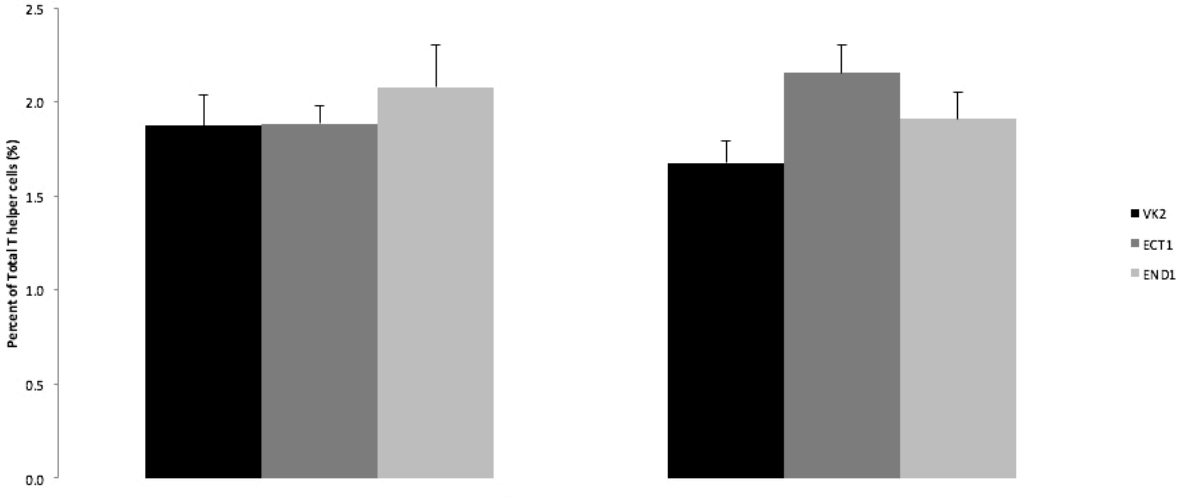
Fig. 5.2. Each cell line does not differ in $CD3^+CD4^+$ T cells in the presence of semen. (**A**) PBMCs from transwells were collected at 4 h from the basolateral chambers from mock and semen wells of each cell line. The number of $CD3^+CD4^+$ T cells is expressed as a percentage of the total Lymphocyte population found in the harvested PBMCs. (**B**) The surface expression density of CD4 on $CD3^+CD4^+$ T cells co-cultured with ECT1 cells is lower compared to VK2 and END1 cells. MFI is median fluorescence intensity. * is $p<0.05$.

5.4.5 Compared to $CD3^+CD4^+$ T helper cells co-cultured with VK2 or END1 cells, CCR5 surface expression is elevated on $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells

The evaluation of CD4 on T helper cells provided insight to some of the effects semen had on immune cells that were co-cultured with representative cell lines of the FRT. However, the next step was to evaluate what effects semen had on the percent of $CCR5^+$ T helper cells available and the surface expression of CCR5.

The percent of $CD3^+CD4^+CCR5^+$ T cells available were comparable across all three regional cell lines in either non-semen exposed or semen exposed wells (Fig. 5.3A). Although $CD4^+CD3^+$ T cells made up about 50% of the total lymphocytes that were present (Fig. 5.2A), the numbers of T cells that were also $CCR5^+$ in this population were relatively small (~2%) (Fig. 5.3A). Semen had no effect on the size of this cell population (Fig 3A).

Figure 5.3A



Epithelium	+	+
25% Semen	-	+

Figure 5.3B

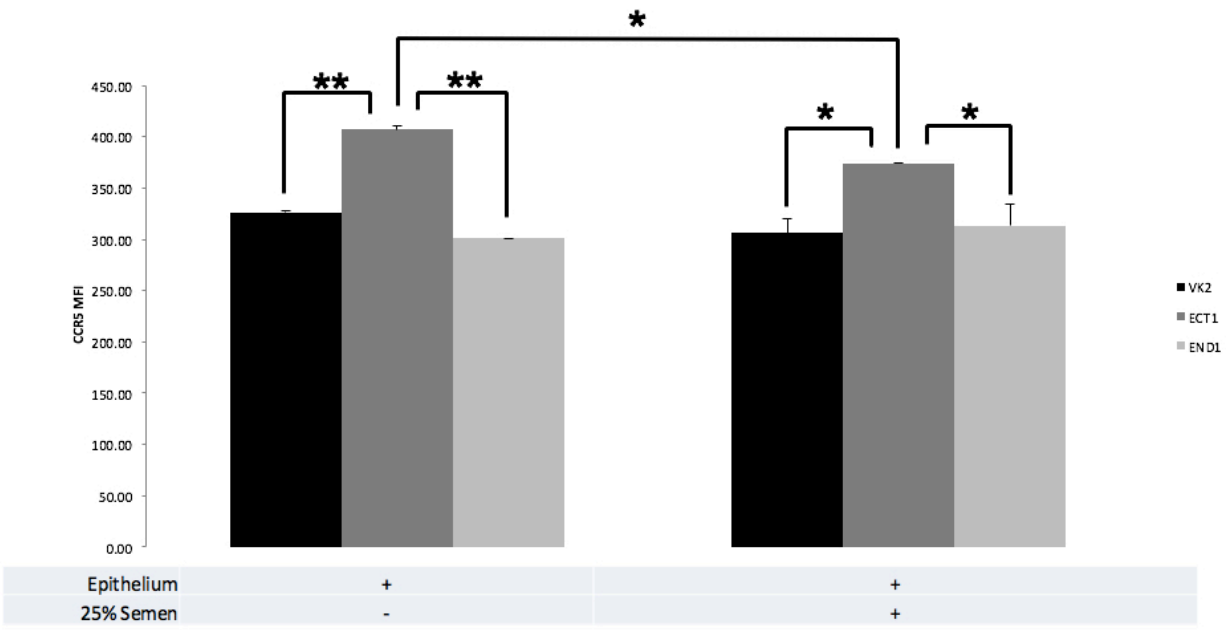


Fig. 5.3. In the presence of semen, $CD3^+CD4^+CCR5^+$ T cells co-cultured with ECT1 cells have an increase in CCR5 surface expression. (**A**) PBMCs were collected from the basolateral chamber of transwells at 4 h post 25% semen exposure from mock and semen wells of each cell line. The number of $CD3^+CD4^+CCR5^+$ T cells is expressed as a percentage of the total $CD3^+CD4^+$ T cells. There is no difference in $CD3^+CD4^+CCR5^+$ T cells between the three cell lines of the FRT. (**B**) The surface expression density of CCR5 is increased on $CD3^+CD4^+CCR5^+$ T cells co-cultured with ECT1 cells compared to VK2 and END1 cells, which are similar. MFI is median fluorescence intensity. * is $p<0.05$.

The levels of cell surface expression of CCR5 on T helper cells co-cultured with VK2 and END1 cells did not differ from non-semen exposed wells to semen exposed wells (Fig. 5.3B). In fact, the only difference in cell surface density of CCR5 was when $CD3^+CD4^+$ T helper cells were co-cultured with ECT1 cells and exposed to semen (Fig. 5.3B) CCR5 had a higher density on T helper cells co-cultured with ECT1 cells than when compared to T helper cells co-cultured with VK2 or END1 cells, both in non-semen exposed wells and semen exposed wells (Fig. 5.3B). In non-semen exposed wells, the surface density of CCR5 was 24.8% and 35.2% greater on $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells than when $CD3^+CD4^+$ T helper cells were co-cultured with VK2 or END1 cells, respectively (Fig 3B). In semen exposed wells the surface density of CCR5 was 22.2% and 19.3% greater on $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells than when $CD3^+CD4^+$ T helper cells were co-cultured with VK2 or END1 cells, respectively (Fig 3B). Interestingly, when exposed to semen $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells had a significant 8% reduction in cell surface density of CCR5 compared to when the same cells were non-semen exposed (Fig. 5.3B).

5.4.6 Semen increases CXCR4 surface expression on $CD3^+CD4^+$ T helper cells co-cultured with END1 cells

Just as with CCR5 attention to CXCR4 was the next step. By evaluating semen's effect on HIV-1 viral co-receptors, conclusions about viral tropism affected by semen could be drawn. Therefore, the percent of $CD3^+CD4^+CXCR4^+$ T cells as

well as surface expression of CXCR4 on $CD3^+CD4^+$ T helper cells co-cultured with each cell line was elucidated.

$CD3^+CD4^+CXCR4^+$ T helper cells co-cultured with VK2 cells decreased by a significant 5% in semen exposed wells when compared to non-semen exposed wells (Fig. 5.4A). However, in the absence of semen, the percent of $CD3^+CD4^+$ T helper cells positive for CXCR4 is about the same when $CD3^+CD4^+$ T helper cells were co-cultured with every cell line (Fig. 5.4A). Therefore, semen seems to preferentially decrease $CXCR4^+$ T helper cells in only $CD3^+CD4^+$ T helper cells co-cultured with VK2 (Fig. 5.4A).

The surface expression of CXCR4 on $CD3^+CD4^+$ T helper cells co-cultured with END1 cells significantly increased by about 36.3% from non-semen exposed wells to semen exposed wells (Fig. 5.4B). In fact, in semen exposed wells surface expression of CXCR4 was significantly higher on $CD3^+CD4^+$ T helper cells co-cultured with END1 cells versus $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells by 38.6% (Fig. 5.4B)

5.4.7 Semen increases R5 HIV-1 infections in $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells but decreases infections in $CD3^+CD4^+$ T helper cells co-cultured with VK2 or END1 cells

In order to determine if semen has some role in helping R5 viruses enter and infect cells over X4 viruses we performed a pseudovirus infection on PBMCs co-cultured with epithelial cells exposed to semen. After each cell line was exposed to semen, the PBMCs were immediately harvested and challenged in an *in vitro*

infection model with R5 or X4 pseudovirus for 4 h. The cells recovered for 48 h and were then assayed them for enhanced green fluorescent protein (eGFP) production via flow cytometry. $CD3^+CD4^+$ T helper cells co-cultured with VK2 cells had a reduction in percent of cells infected by R5 viruses (9.57%) when semen exposed wells were compared to non-semen exposed wells (Fig. 5.5A). Similarly, $CD3^+CD4^+$ T helper cells co-cultured with END1 cells displayed a 5.3% reduction in the percent of $CD3^+CD4^+$ T helper cells infected when semen exposed wells were compared to non-semen exposed wells (Fig. 5.5A). Unlike the other cell lines, $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells saw an increase in $CD3^+CD4^+$ T helper cells infected by R5 viruses by 24.5% (Fig. 5.5A). Therefore, semen appeared to preferentially enhance R5 infection in only $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells (Fig. 5.5A).

Figure 5.4A

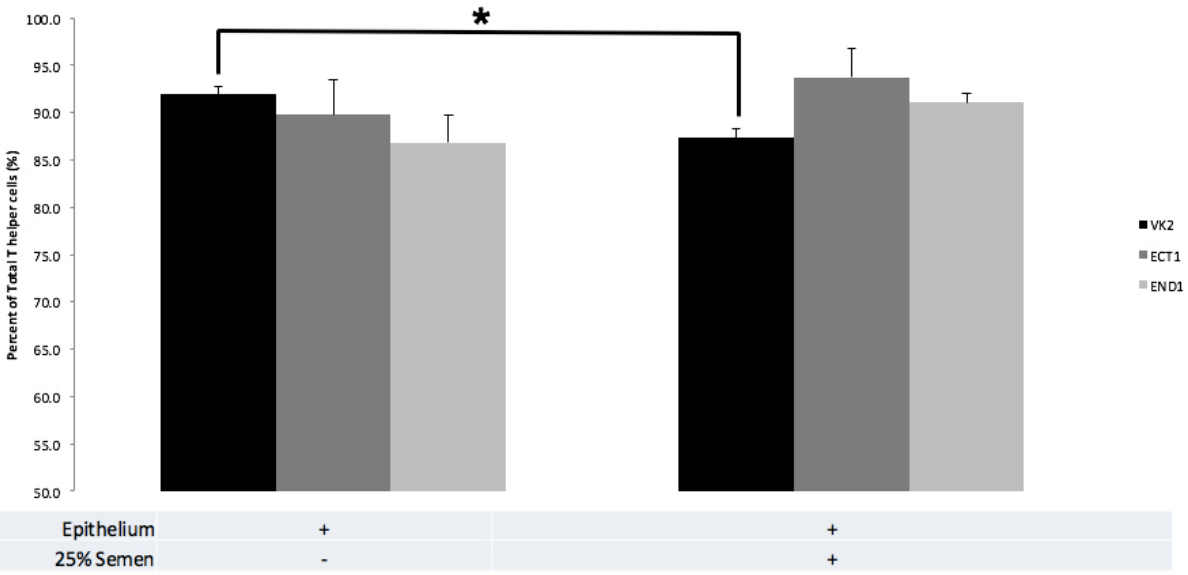

*
100.0
95.0
90.0
85.0
80.0
75.0
70.0
65.0
60.0
55.0
50.0
Percent of Total T helper cells (%)
VK2
ECT1
END1
Epithelium + +
25% Semen - +

Figure 5.4B

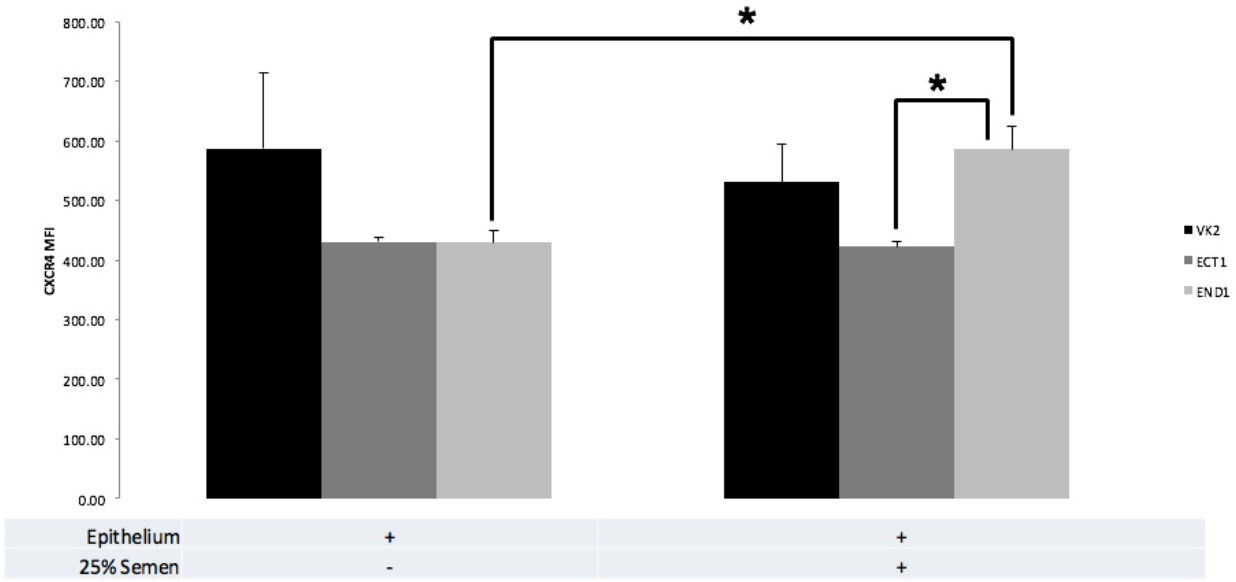
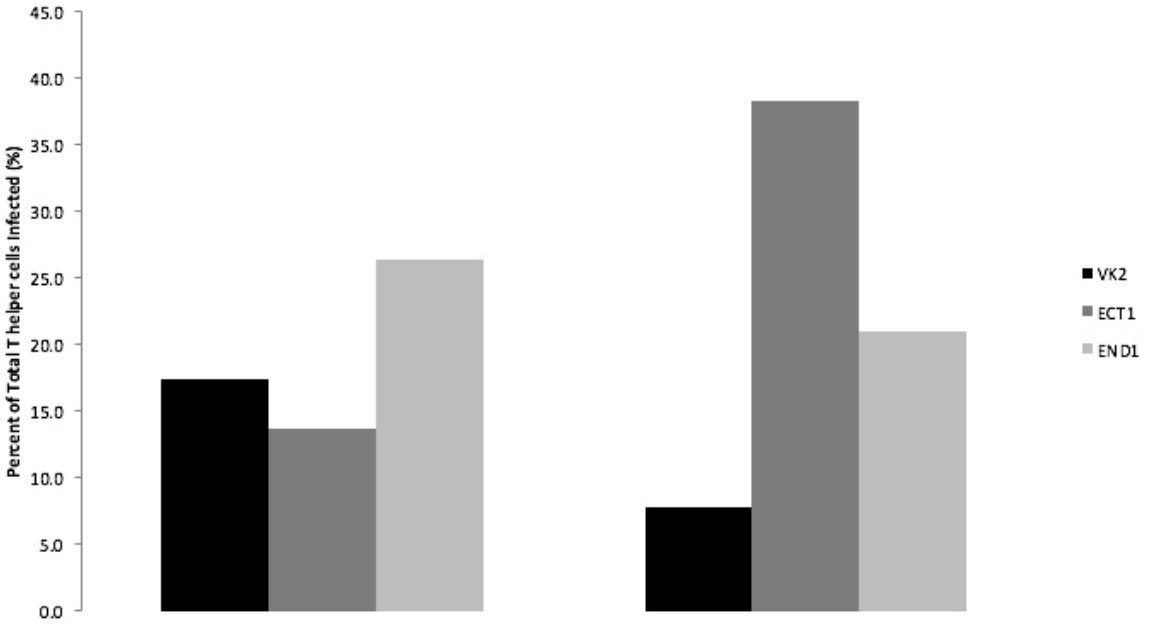


Fig. 5.4. In the presence of semen, $CD3^+CD4^+CXCR4^+$ T cells co-cultured with END1 cells have an increase in CXCR4 surface density. (**A**) After exposure to 25% semen for 4 h PBMCs from basolateral chambers of mock and semen transwells were collected from each cell line. The number of $CD3^+CD4^+CXCR4^+$ T cells is expressed as a percentage of the total $CD3^+CD4^+$ T cells. In the absence of semen $CD3^+CD4^+CXCR4^+$ T cells are about even when co-cultured with each cell line, however in the presence of semen ECT1 and END1 cells have more $CD3^+CD4^+CXCR4^+$ T cells. (**B**) The surface expression density of CXCR4 increases on $CD3^+CD4^+CXCR4^+$ T cells co-cultured with END1 cells but remains the same with VK2 and ECT1 cells. MFI is median fluorescence intensity. * is $p<0.05$.

The level of eGFP expression correlates to the level at which the infected cell supports higher levels of long term terminal repeat (LTR) activity and, by extension viral gene expression. This would suggest higher levels of replication, but because the pseudovirus is incapable of continued replication these data can't be verified. Despite this, however, it was clear that $CD3^{+}CD4^{+}$ T helper cells co-cultured with ECT1 cells promote greater levels of replication in semen exposed wells then non-semen exposed wells by 20% (Fig 5B).

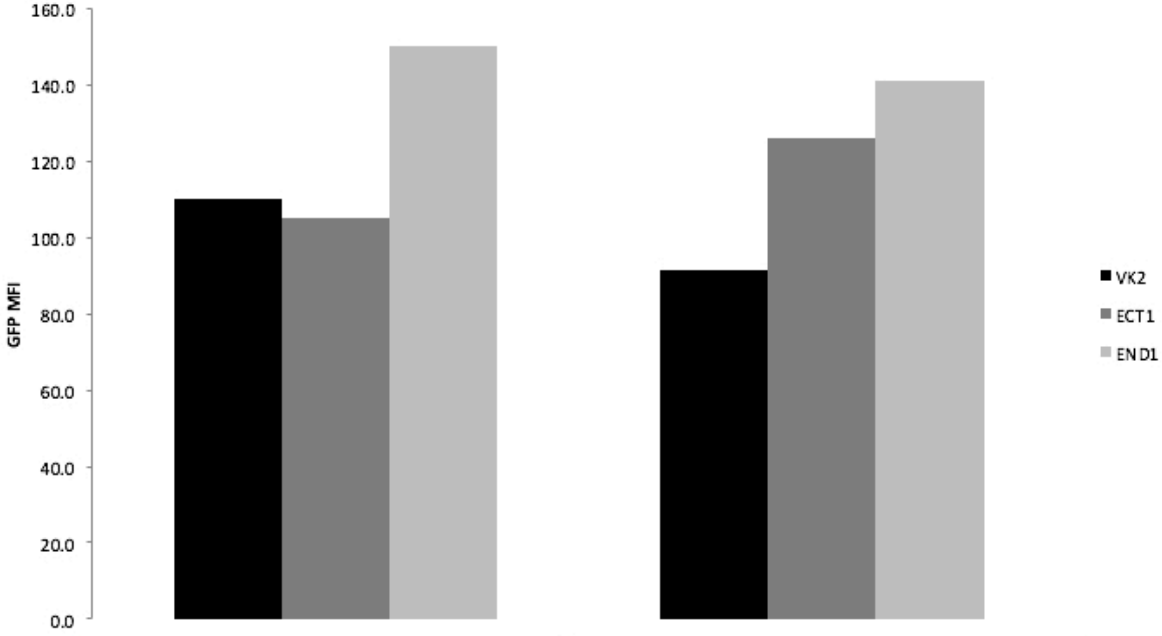
Unlike in R5 infections, semen enhanced all X4 infections by 5.9%, 6.5%, and 10.5% for $CD3^{+}CD4^{+}$ T helper cells co-cultured with VK2, ECT1, and END1 cells, respectively (Fig. 5.6A). For $CD3^{+}CD4^{+}$ T helper cells co-cultured with VK2 cells the levels of replication remained constant (Fig. 5.6B). But for $CD3^{+}CD4^{+}$ T helper cells co-cultured with ECT1 and END1 cells the replication levels of X4 viruses reduced by 13% and 10.5% (Fig. 5.6B).

Figure 5.5A



Epithelium	+	+
R5 Virus	+	+
25% Semen	-	+

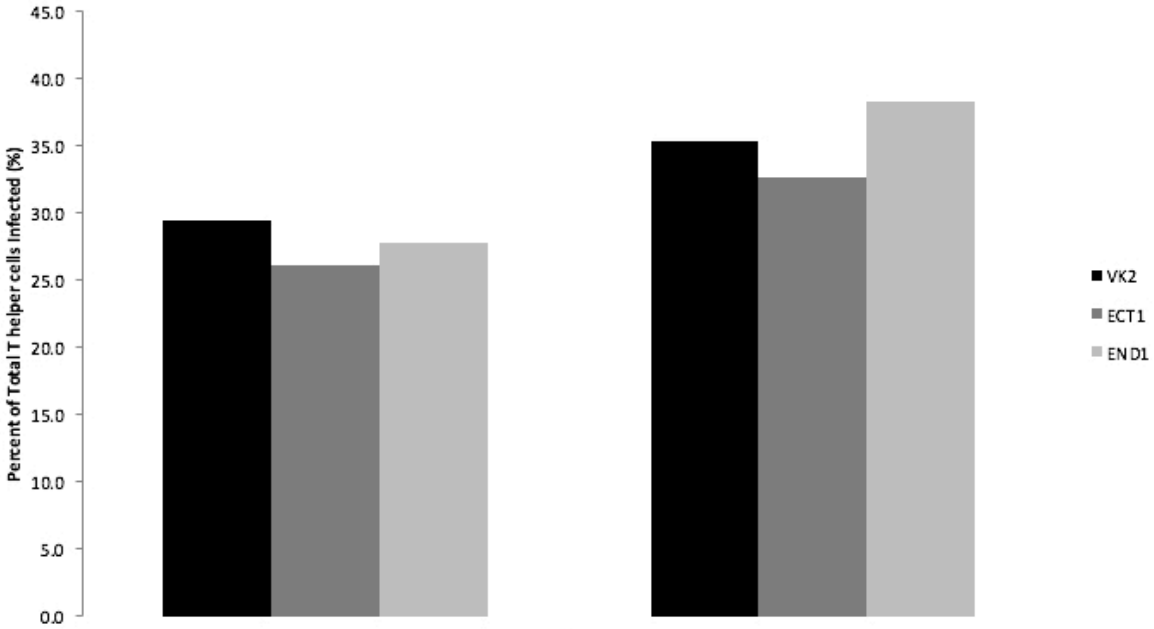
Figure 5.5B



Epithelium	+	+
R5 Virus	+	+
25% Semen	-	+

Fig. 5.5. Semen increases R5 viral infections in $CD3^+CD4^+$ T cells co-cultured with ECT1 cells reduces R5 infections in the other cell lines. (**A**) PBMCs from mock and semen exposed wells from each cell line were collected 4 h after 25% exposure and immediately challenged with a pseudovirus infection for 4 h. The cells were then washed and given 48 h recovery. The number of cells infected is expressed as a percent of total $CD3^+CD4^+$ T cells. $CD3^+CD4^+$ T cells co-cultured with ECT1 cells had more cells infected with pseudovirus than of those co-cultured with VK2 or END1 cells. **(B)** $CD3^+CD4^+$ T cells from ECT1 cells also have more viral gene expression in each infected cell versus the other cell lines of the FRT when exposed to semen.

Figure 5.6A



Epithelium	+	+
R5 Virus	+	+
25% Semen	-	+

Figure 5.6B

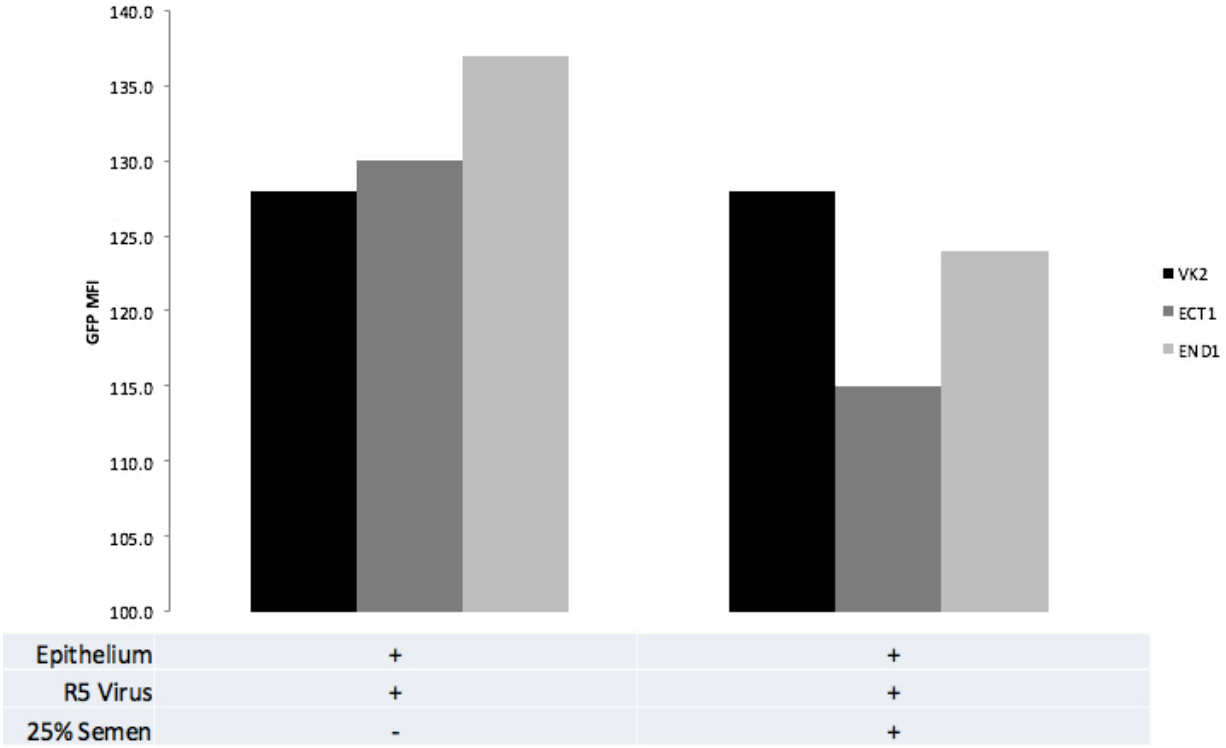


Fig. 5.6. X4 infections are increased in the presence of semen across each cell line (**A**) PBMCs from mock and semen exposed wells from each cell line were collected 4 h after 25% exposure and immediately challenged with a pseudovirus infection for 4 h. The cells were then washed and given 48 h recovery. The number of cells infected is expressed as a percent of total $CD3^{+}CD4^{+}$ T cells. In each cell line of the FRT, in the presence of semen, X4 infections are increased. (**B**) Viral gene expression is decreased in ECT1 cells but remains the same in the VK2, despite an increase in infections seen in VK2 cells.

5.5 Discussion

HIV-1 transmission from male-to-female is a complicated process that involves multiple aspects of reproductive biology in both the male donor and female recipient. The male reproductive tract can influence which genetic variants out of a total become found in semen [79, 132]. From here, this pool of variants diminishes as semen is deposited in the FRT [132]. Indeed, these multiple checkpoints are part of the bottleneck hypothesis, which states that at points during transmission of HIV-1, there exist filtering steps that select for one or more HIV-1 genetic variants [130]. This selection process eventually leads to the nearly exclusive transmission of R5 viruses [131, 132].

Of the checkpoints, the most stringent would be when semen comes in contact with the FRT [131, 132]. And most concepts in the literature take this to be the FRT, as a whole [130]. However, what we have shown here is that each region of the FRT acts as a different checkpoint independently of one another. This notion posits that the bottleneck hypothesis is a lot more complicated than previously thought, insofar the different regions of the FRT would have different mechanisms to filter out and/or select for specific genetic variants out of the pool they are presented with.

In fact, looking at the cytokine profiles generated in this study we can see that no two region responded the same to stimuli. When only semen is cultured with each region of the FRT, we see a distinct profile in our analytes for each region [84]. IL-8 was one of, if not the only, cytokine that was constitutively upregulated in each region. However, IL-8 is a potent chemotactic agent for immune cells.

Indeed, the biphasic effect of semen sees an induction of a pro-inflammatory environment early on when semen comes in contact with the FRT [100, 102, 116, 126, 127], therefore IL-8 will be one of the first cytokines upregulated [84]. When semen comes in contact with the FRT, the initial steps to pregnancy are started [5, 59, 77, 99, 125] and in those initial steps IL-8 is one important cytokine because of its chemotactic properties but also because of its role in angiogenesis and embryo development [104].

TNF-α is another potent mediator of a pro-inflammatory environment and we did see a doubling of TNF-α concentrations across all regions of the FRT. The same can be said of any analyte in our panel. Though, interestingly enough, we see that END1 cells were the most responsive to semen alone. END1 cells also had the largest response with respect to IL-6, IL-17A, and RANTES. These chemokines are also involved in the beginning stages of pregnancy, with IL-6 have tissue remodeling properties [67, 76].

Initially, we thought that ECT1 cells would be the most responsive to semen but what we see here was not the same. However, ECT1 cells was the most responsive region when co-cultured with PBMCs. Indeed, the cross-talk between ECT1 cells and the underlying peripheral blood cells seemed to attenuate the pro-inflammatory response. As stated before, across all cell lines, the concentrations of chemoattractant cytokines was greater than that of the other cytokines, save IL-8. This is especially true in ECT1 cells, where the fold-changes for analytes were higher than the other two cell lines. Again, this may be due, in part, to the fact that the transformation zone is biologically more active than the other areas of the FRT

and routinely sees an influx and turnover of immune cells that then drain to other parts of the body [103]. Therefore, ECT1 cells will appear to be more responsive to peripheral blood cells in the basolateral chamber than the other two cell lines [95, 103, 104].

When the three FRT derived cell lines are co-cultured with both semen and PBMCs, ECT1 cells still displayed the largest response. Even IL-8, which was the highest reported cytokine in all regions, was the highest in ECT1 cells, with about a 46.5-fold change over non-semen exposed wells. The closest region to this would be the vagina, which is at about a 42-fold change over non-semen exposed cells. However, what was not seen was an over active response, or underactive, response in any condition. Each region had about the same response to each stimulus. This is curious as each cell line has previously been shown to act independently of one another with distinct cytokine patterns (Appendix Chapters 1 and 2). Perhaps the co-culture with peripheral blood cells has skewed the cell lines to mimic one another. Indeed, the ectocervix has the highest abundance of immune cells found in the subepithelium [102], but by co-culturing each cell line with the same amount of PBMCs the results would all mimic one another. If each cell line was co-cultured with their respective amount of immune cells, maybe each cytokine profile would better have reflected what was previously reported (Appendix Chapter 1).

Apical exposure of semen on cell lines derived from regions of the FRT provided an idea of the immune microenvironment produced by each cell line as a response to semen. However, semen did not only modulate responses from an

epithelium, it also transduced its effect across the epithelium to affect peripheral blood cells co-cultured with each cell line. This eventually led to differential infection profiles of those immune cells. When exposed to semen, only $CD3^+CD4^+$ T helper cells co-cultured with END1 cells decreased by 5%. This reduction was most likely due to cell death as the immune microenvironment purported T cell proliferation and expansion. Indeed, PBMC treatment of IL-2 overnight and then placing the treated cells in culture may have induced a rapid turn-over of T cells and this snapshot was at a low point. Because, again, with the kind of immune microenvironment abundant T lymphocytes should flourish [95, 103, 126] . In fact, the high concentrations of IL-8 will further attenuate IL-8 from $CD4^+$ T cells and thus bring about an influx of more T lymphocytes [149]. However, this is a closed system that does not allow for the influx of new, fresh immune cells. Actually, $CD3^+CD4^+CCR5^+$ T helper cells co-cultured with any cell line did not increase or decrease in number unlike the <5% reduction in $CD3^+CD4^+CXCR4^+$ T helper cells co-cultured with VK2 cells. The latter result may have been due to 10% reduction in CXCR4 surface expression when $CD3^+CD4^+$ T helper cells co-cultured with VK2 cells and exposed to semen.

In fact, the only differences we saw were about the surface expression of the HIV-1 co-receptors. CD4 is significantly less expressed on $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells when compared to the rest of the cell lines. This would suggest that HIV-1 would might have an easier time entering cells in the transformation zone because a determinative factor is relative abundance of the co-receptors on the surface of target cells [150-152].

Despite the reduction in surface expression of CD4 on $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells, CCR5 surface expression was significantly increased when $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells were exposed to semen when compared to VK2 and END1 cell lines. One explanation may be that ECT1 cells released more interferon alpha (IFN-α) subsequent to semen exposure (Appendix Chapter 1) [84]. IFN-α is known to induce CCR5 expression on T lymphocytes, as well as IL-12 and TNF- α [67, 147, 153, 154]. From previous work we have identified IFN-α as having a greater than 10-fold change over mock when ECT1 cells were exposed to semen, which was significantly higher than VK2 or END1 cells (Appendix Chapter 1). The effects of these three cytokines on T lymphocytes is consistent with our observations.

It seems as though $CD3^+CD4^+$ T helper cells co-cultured with VK2 or END1 cells were similarly affected by the presence of semen. Indeed, the amount of $CD3^+CD4^+$ T helper cells and the surface expression of CCR5 and CXCR4 remained similar to one another throughout this study. There was one exception—CXCR4 surface expression increases on $CD3^+CD4^+$ T helper cells co-cultured with END1 cells when exposed to semen. CXCR4 has been shown to be involved in organogenesis and fetal development [155]. Therefore, the increase in receptors on immune cells in the upper portion of the female reproductive tract would be consistent with the fact that the upper FRT undergoes remodeling due to the presence of semen, whose main function is to induce pregnancy and embryo development.

In fact, by semen increasing CXCR4 on $CD3^+CD4^+$ T helper cells co-cultured with END1 cells, we should have seen an increase in X4 HIV-1 infection in the endocervix. Actually, we saw that the other two regions also follow suit with having an increase in X4 HIV-1 infection in the presence of semen. However, the most interesting result was that in the presence of semen R5 HIV-1 infections decreased in $CD3^+CD4^+$ T helper cells co-cultured with VK2 or END1 cells but increased in $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells. ECT1 R5 HIV-1 infections in the presence of semen significantly increased to just about the highest percentage of all infections performed.

When $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells were exposed to semen, surface expression of CD4 was decreased, CCR5 surface expression was increased. These two factors would make for the perfect situation for R5 viruses to enter and infect $CD3^+CD4^+$ T helper cells [150-152, 154]. Perhaps these factors are what drove the increase in R5 infections in only ECT1 cells.

In conclusion, we have identified that the regions of the FRT are individualized and acted in an individual manner, yet the basic response of each region was very similar. However, the ECT1 cells did seem to be more responsive than VK2 cells, with END1 cells being the most responsive to semen and ECT1 cells being the most responsive to semen when immune cells were present. Furthermore, when semen and immune cells were present, together, ECT1 cells enhanced infection by R5 HIV-1, where VK2 and END1 cells did not. The differences in $CD3^+CD4^+$ T helper cells co-cultured with ECT1 cells versus $CD3^+CD4^+$ T helper cells co-cultured with VK2 or END1 cells were attributable to

semen exposure. And it was those differences that created the kind of $CD3^+CD4^+$ T helper cells to be infected by R5 viruses: slight reduction in CD4 surface expression with an upregulation in CCR5 surface expression [150-152, 154]. The effect the FRT plays in the bottleneck hypothesis can be broken down to region-specific differences attributed to their specific responses to semen. The immunological response elicited by each barrier will further modulate peripheral blood cells to make them more susceptible to R5 infections in the ectocervix only.

Further elucidation of this pathway in the ectocervix using primary tissue and infectious virus will provide new insights into what specific factors are enhancing R5 infection over X4 infection. Understanding all key aspects may provide new targets for therapy or prevention medicines.

Chapter 6

The mechanical and biological effect of semen on the female reproductive tract

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6.1 Abstract

When semen comes in contact with the female reproductive tract, there is an almost immediate mechanical response by the epithelial barrier. This response is a reaction to protein and metal-ion factors found in semen, which reduces permeability of the barrier through upregulation of tight junctions between adjacent cells. Closing of the paracellular spaces prevents semen, its constituents, and pathogens from traversing the barrier. Furthermore, semen also elicits a biological response from the epithelium by way of cytokine and immunomodulatory factors. In doing so semen modulates female immunity to ensure the clearance of excess and abnormal sperm while promoting the acceptance of healthy sperm. This is known as the biphasic effect of semen. The inflammation associated with semen interaction starts the process and is carried out by transforming growth factor beta (TGF-β) releasing interleukin 6 (IL-6) and granulocyte macrophage colony-stimulating factor (GM-CSF) from the epithelium. These cytokines will induce an influx of immune cells to the cervix where cell mediated immunity will provide the necessary components to clear said sperm. However, this initial inflammation is soon abated to tolerance by TGF-β released from the epithelium. This promotes the expansion and proliferation of T regulatory cells (Tregs), which act to dampen inflammation. Dampening the innate immune response ensures the survival of healthy sperm to establish pregnancy and embryo development. However, modulating the innate immune system has downstream effects involved in pathogen transmission from male-to-female.

6.2 Introduction

There is a continuing need to elucidate interactions between semen and the female reproductive tract (FRT). Only recently has semen been thought of as more than an inert vehicle for sperm delivery [29]. Recent studies, including our own, have characterized semen as a biologically active substance that contains a myriad of factors such as sugars, whole cells, cytokines, and other immunomodulatory factors [9, 33, 49, 51, 59, 61, 115, 120]. The concentrations of these factors differ greatly from one male to the next and are influenced by factors such as age, disease, diet, and lifestyle [9, 33, 42, 49, 120]. The concentrations of these factors can also influence downstream events, such a pregnancy and embryo implantation [9].

Of these factors, the most prevalent is transforming growth factor beta (TGF-β) [4]. The interaction of TGF-β and the FRT has shown that TGF-β favors embryo development and implantation through modulation of the local immune immune environment [4, 9]. Cervical epithelial cells were shown to increase granulocyte macrophage stimulating factor (GM-CSF) and interleukin 6 (IL-6) when exposed to TGF-β [4], which initiates the necessary local inflammatory response in the female for fetal development [84]. TGF-β originates in the seminal vesicle of the male, where it is secreted into semen. However, the function of seminal vesicle is not just for TGF-β supplementation but for other factors as well, such as prostaglandin E_2 (PGE_2) [80]. PGE_2 also helps control pregnancy through dampening of the female innate immune response [29, 81, 87].

The initial inflammation associated with semen deposition in the FRT is needed to clear abnormal sperm and excess sperm. However, if left unchecked, most of, if not all, the sperm will be cleared [9]. Therefore, factors like PGE_2 work to dampen the immune response by inhibiting monocyte derived cytokines and increasing T lymphocyte proliferation into T regulatory (Treg) phenotypes [81]. This allows for not only paternal antigens to survive, but also developing fetal antigens [9]. Any abnormalities in semen content could lead to dysregulation of one of the functions of semen, such as fetal development and embryogenesis [9].

Therefore, these factors have lasting impacts on the tissues they come in contact with and nowhere else is this more prevalent than when semen comes in contact with the FRT. Interactions between semen and the FRT elicit mechanical responses as well as biological responses [29] (Appendix Chapters 1 and 2). The goal of this review is to establish a base line for semen's effect on the FRT and what implications these could have on immune modulation and pathogen transmission.

6.3 Semen reduces paracellular permeability through upregulation of tight junctions

Mechanical responses to semen deposition in the FRT largely include tightening of paracellular spaces through tight junction upregulation [156-159]. When semen comes in contact with reproductive epithelia, tight junctions increase which leads to the reduction in permeability across the epithelium [159]. In one study, after direct exposure to semen for 4 h, the transepithelial electrical

resistance (TEER) was measured and there was a large increase over basal levels (Appendix Chapter 2). TEER is a direct measurement to tight junctions through measuring the resistance of a current passing in-between adjacent cells from the apical side to the basolateral side. Therefore, if the resistance is higher, measuring as a higher TEER, then there is greater resistance between cells—signifying a greater abundance of tight junctions [157]. These tight junctions were shown to upregulate in as early as 5 min post semen exposure, which is too rapid for protein transcription and translation (Appendix Chapter 2).

Therefore, there is some other mechanism behind the quick upregulation of tight junctions. This effect is due to the constituents of semen (Appendix Chapter 2). As stated before semen is more than an inert vehicle for sperm delivery. The factors that make up semen have been extensively studied and it has been identified that a combination of calcium, lipids, and soluble proteins are responsible for the increase in tight junctions [9, 29, 156, 160] (Appendix Chapter 2). When polarized epithelia were exposed to physiological concentrations of metal-ions found in semen it was found out that each increased TEER by at least 113%-fold over non-treated wells [161] (Appendix Chapter 2). During co-incubation of semen with ethylenediaminetetracetic acid (EDTA), a known metal-ion chelator, the increase in TEER was abrogated to basal levels (Appendix Chapter 2).

In fact, ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetracetic acid (EGTA) is known to chelate calcium, specifically, and when co-incubated with semen there was a reduction in TEER from semen-induced levels back down to basal levels (Appendix Chapter 2). This reduction of calcium, in specific, suggests

that calcium is the main metal-ion in semen affecting tight junctions (Appendix Chapter 2). It is known that calcium induces tight junction formation through upregulating zonula occludin 1 (ZO-1) [160, 162], a scaffolding protein that acts as an accessory to tight junction protein complexes of occludins and claudins [163]. Staining for ZO-1 one sees a mesh-like network at the periphery of each cell (Appendix Chapter 2). However, when the reproductive cell lines were co-incubated with EDTA/EGTA and then stained for ZO-1 the network all but dispersed, suggesting that without calcium the tight junction network could not form, allowing paracellular transport of foreign antigens (Appendix Chapter 2). Of course, calcium is not the only major player in this phenomenon [27, 161, 162]. When semen was heat-inactivated, by any means, there was a significant, partial reduction in TEER suggesting that there is a protein component also acting on on the regulation of tight junctions (Appendix Chapter 2).

By closing the paracellular space between cells in the FRT semen also prevents any foreign particles that may be found in the seminal fluid from easily crossing the epithelial barrier (Appendix Chapter 2). In an experiment to determine permeability of the epithelia, dextran conjugated to fluorescein isothiocyanate (FITC) was apically applied to epithelia grown in a polarized manner on a transwell after semen exposure (Appendix Chapter 2). The amount of FITC-dextran that diffused through the epithelium compared to input concentration denoted permeability, and, in the presence of semen, permeability was reduced (Appendix Chapter 2). Using EDTA, as a negative control for tight junction formation,

significantly increased permeability, suggesting that tight junctions were responsible for the barrier function the epithelia [164] (Appendix Chapter 2).

The tight junctions act like links in a fence [164] (Appendix Chapter 2), which keep foreign particles from easily crossing the epithelium. However, the effect semen elicits on the FRT is not just mechanical. When semen comes in contact with the FRT it elicits a biphasic effect [9, 28] (Chapter 3), which is the induction of inflammation that is quickly abated to tolerance [5, 77, 87, 99, 125]. The biological importance of the biphasic effect is to establish pregnancy because when seminal antigens come in contact with the FRT they are viewed as foreign and would otherwise be targeted for removal by the female immune system [9, 29].

6.4 Seminal constituents activate the innate immune response in the female reproductive tract

The initial inflammation associated with semen exposure upregulates tumor necrosis factor alpha (TNF-α) [9, 28, 100, 106, 126], which is a potent pro-inflammatory molecule. This cytokine will then attenuate inflammation through monocytes. In fact, TNF-α, as well as IL-6, interleukin 8 (IL-8), interleukin 1 alpha/beta (IL-1α/IL-1β), and GM-CSF, will all further enhance the pro-inflammatory cascade by acting on monocytes and macrophages [28, 70, 87, 99, 102, 114, 126] (Chapter 3). The macrophages, with interferon gamma (IFN-γ), interact with mature dendritic cells (DC), which can activate natural killer cell (NK) populations with interleukin 12 (IL-12) (Chapter 3). DCs can also interact with T lymphocytes to increase pro-inflammatory signaling as well as increase T cell

proliferation and migration (Chapter 3). The influx of T lymphocytes to the site of semen deposition causes naïve T cells to differentiate into Th_1, Th_2, or Th_{17} cells (Chapter 3). Each of these T cell subsets produce more pro-inflammatory cytokines that further extend inflammation.

However, semen exposure in the FRT also elicits interleukin 10 (IL-10) and TGF-β to be released from the epithelium [4, 9, 29, 67, 104, 124] (Chapter 3). As inflammation occurs, the concentrations of IL-10 and TGF-β are influencing $CD4^+$ T cells to differentiate into T regulatory cells (Treg) [109, 124, 165, 166]. The anti-inflammatory Tregs will shut down NK cells, Th_1, Th_2, and Th_{17} subsets by producing more IL-10 and TGF-β (Chapter 3). Tregs are also auto-inducing, in that they add to overall growing concentration of IL-10 and TGF-β, which will further attenuate Treg proliferation [78, 102, 125]. By having more Tregs present in the FRT the initial inflammation associated with semen will be quickly resolved to tolerance. In a mouse model, the generation of Tregs has been shown to be induced early after coitus [5, 77, 124]. The need for tolerance comes with the initial responsibility of semen, which is to survive the FRT to fertilize an ovum in the uterus. By dampening the immune response in the female, semen ensures that paternal antigens are not targets for degradation [9, 29].

The biological effect of semen in the FRT is not limited to release of TNF-α and TGF-β from the epithelium [103, 104]. Indeed, the immune response to semen in the FRT is characterized by a much more pro-inflammatory nature, especially within 4 h of exposure (Appendix Chapter 1). Therefore, it is important to

understand what the immune microenvironment looks like in the FRT because this may have further downstream effects.

Although semen elicits a pro-inflammatory response from the FRT as a whole, the tract is divided into specific regions; the vagina, the cervix, and the uterus. Each region has its own specific response to semen and other constituents found in the FRT [29, 103, 104, 126] (Appendix Chapters 1 and 2). The vagina, characterized by multiple layers of a squamous epithelium is the least internalized region. The vagina routinely comes in contact with a variety of foreign stimuli and because of this has the second most abundant concentration of immune cells in the FRT [102, 103]. Regular sloughing and replenishing of the top epithelial layers is common in the vagina and helps keep out foreign antigens and pathogens. This is especially helpful during semen clearance, as the vagina is first and most likely region to come in physical contact with semen [9, 29].

Using the VK2-E6E7 cell line to represent the vagina, the measured output of 30 different cytokines in a multiplex experiment revealed that the majority of cytokines expressed by the cell line when exposed to semen had roles in cell signaling and attenuating the pro-inflammatory environment [29] (Appendix Chapter 1). Interleukin 7 (IL-7) and interleukin 15 (IL-15) were expressed at greater than a 10-fold change over mock, which was defined as no semen exposure (Appendix Chapter 1). Vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and basic fibroblast growth factor (FGF-basic) were also expressed in the range of 5.1-10-fold change over mock (Appendix Chapter 1). Aside from those, interferon alpha (IFN-α), IL-12, IL-6, IFN-γ, and IL-1β were all

moderately expressed from the epithelium in concentrations up to a 5-fold increase over mock (Appendix Chapter 1). Taken together, the use of VK2 cells suggests that the vagina is rich in growth factors to promote the maturity of immune cells that are found in the subepithelium and immune cells being recruited to the subepithelial space [29] (Appendix Chapter 1).

The second area semen comes in contact with is the cervix, which is in between the vagina and the uterus. The cervix is divided into two distinct regions with the lower portion termed the ectocervix and the upper portion referred to as the endocervix. The two cervical regions act differently from one another, with respect to immunity [103]. Indeed, the ectocervix, with the vagina, is considered to be more representative of the lower FRT, while the endocervix is thought to be similar to the upper FRT. The ectocervix, like the vagina, is also characterized by layers of squamous epithelia whereas the endocervix is a single, columnar epithelium. Where the ectocervix meets the endocervix there is an immune microenvironment called the transformation zone—which is routinely represented as the ectocervix as a whole [102, 103, 111]. The transformation zone is unique and important for the FRT in that it has the highest concentration of all immune cells and and is thought of as the central hub for cell mediated immunity [103]. Therefore, the initial inflammation and abatement to tolerance is thought to begin in this region of the FRT.

When apically exposing the cell line ECT1/E6E7, which represents the ectocervix, to semen the activation of the transformation zone is clear (Chapter 4) (Appendix Chapter 1). Just like the response of the VK2 cell line, the ECT1 line is

pro-inflammatory in nature with a large response in growth factors (Chapter 4) (Appendix Chapter 1). Of the pro-inflammatory cytokines IL-12 and IL-6 are increased in concentrations over 10-fold when compared to mock (Chapter 4) (Appendix Chapter 1). IFN-α and IL-1β were increased at about a 2-5-fold change over mock, while IFN-γ was increased up to about 10-fold over mock (Chapter 4) (Appendix Chapter 1). The dramatic increase in these pro-inflammatory cytokines, as compared to the other regions of the FRT, reinforces the idea that the transformation zone is the central hub for cell mediated immunity [102-104].

The ECT1 cell line did not just increase in pro-inflammatory cytokines but also in growth factors and cell signaling molecules (Chapter 4; Appendix Chapter 1). The greater than 10-fold increase over mock of IL-7, VEGF, and IL-2r suggests that an influx of immune cells from peripheral blood will be recruited and matured quickly (Appendix Chapter 1). The 5-10-fold change over mock concentrations of HGF, IL-15, and granulocyte colony-stimulating factor (G-CSF) further attenuate the proliferation of mature immune cells (Appendix Chapter 1). In fact, the ECT1 cell line was the only to report an increase over mock of G-CSF (Appendix Chapter 1), with the other cell lines having a non-detectable concentration further supporting the fact that the transformation zone is the central hub of immunity in the FRT [103].

Unlike the vagina and the ectocervix, the endocervix is not routinely exposed to foreign stimuli from the outside environment. As a result, the endocervix is mostly a sterile environment. When compared to the vagina and ectocervix, the endocervix has the lowest concentration of immune cells [102].

Perhaps these two facts are one reason why the endocervix is mostly non-responsive to semen (Chapter 5; Appendix Chapter 1). Indeed, when using the END1/E6E7 cell line to represent the endocervix, the END1 cells did not produce concentrations of IFN-γ and IL-1β over detectable limits (Appendix Chapter 1). Furthermore, the only pro-inflammatory cytokines that were increased over mock were I-6, IL-12, and IFN-α, with 2-5-fold changes and up to 1.9-fold change, respectively (Appendix Chapter 1). This data reports that the END1 cell line has a reduced pro-inflammatory response to semen exposure (Appendix Chapter 1).

Looking at the same factors for growth and cell signaling as the other two cell lines, it is clear that the END1 cell line is not very responsive (Appendix Chapter 1). Where the other cell lines had a greater than 10-fold change over mock of IL-7, IL-15, VEGF, and IL-2r the END1 cell line produced no more than a 1.9-fold change of each, except for VEGF, which was between the range of 2-5-fold change over mock (Appendix Chapter 1). Where the VK2 and ECT1 cell lines had an increase in concentration over mock for HGF, the END1 cell line had no detectable limit (Appendix Chapter 1). Therefore, the END1 cell line, and by extension the endocervix, does not seem to rapidly respond to semen (Appendix Chapter 1). Taken further the lower FRT is more responsive than the upper FRT [103, 104, 111] (Chapter 5).

The response to semen shows that the different regions of the FRT act distinctly (Chapter 5) (Appendix Chapters 1 and 2). However, the lower FRT appears to have the same response, with minor differences [103, 104]. Despite these differences the entirety of the FRT has been reported to have a pro-

inflammatory response to semen (Chapter 5) (Appendix Chapter 1). Again, this is the first step in the innate immunity of the female, which is to target foreign antigens for degradation [99, 104]. Only through tolerance induction does the female immune system start to “recognize” sperm as “self” [9, 29]. When cytokines are released from the epithelium, they interact with immune cells that are already found in the subepithelium of the FRT, which include NK cells, macrophages, B lymphocytes, T lymphocytes, granulocytes, and DCs [5, 28, 67, 84, 87, 103, 104, 166].

6.5 Factors released from the female reproductive tract epithelia interact with immune cells present in the subepithelium to induce a pro-inflammatory environment

The interaction of the released cytokines with the present immune cells will further increase cell mediated immunity. As stated before, specific cytokines, like IL-6, IL-1β, and GM-CSF are released from the epithelia and initiate inflammation through interaction with immune cells [9, 28, 29, 58, 84, 116, 167]. These interactions will produce a cascade of different cytokines, which will influence the cytokine milieu in the subepithelium. A study aimed at recapitulating this idea *in vitro* co-cultured peripheral blood mononuclear cells (PBMCs) in the basolateral chamber of a transwell with a representative cell line of a region of the FRT grown on the mesh insert and then apically exposed each cell line to semen (Chapters 4 and 5). Analysis of the basolateral supernatant provided insight into what kind of

cytokines were further elicited from the immune cells already present (Chapters 4 and 5).

In examinations of specific pro-inflammatory cytokines, there were almost no significant differences between all three cell lines, with the exception of TNF-α (Chapter 5). The END1 cell line had significantly more upregulated TNF-α than the VK2 or ECT1 lines (Chapter 5). Of note, however, were IL-8, IL-12, monocyte chemotactic protein one (MCP-1), RANTES, macrophage inflammatory proteins 1 beta (MIP-1β), and macrophage derived chemokine (MDC). IL-8, which had an average of 5.14-fold change over PBMC only wells (PBMC co-culture without apical semen exposure), was the largest fold-change over PBMC only wells (Chapter 8). MCP-1, RANTES, and MDC were all just about doubled in the presence of semen with average fold-changes of 1.25, 0.93, and 1.03, respectively (Chapter 5). The doubling (and then some) of these chemokines leads to influx of more immune cells, specifically monocytes, to the area of inflammation [28].

IL-12 is particularly interesting because the average fold-change of IL-12 across all three cell lines was 0.11 fold-change over PBMCs alone (Chapter 5). What makes this cytokine interesting is that in the PBMCs alone, without semen exposure, the average fold-change was 1.59-fold change over mock (no PBMCs or semen) (Chapter 5). IL-12 is produced in the biphasic effect of semen during both inflammation and tolerance [29]. Indeed, $CD4^+$ T lymphocytes interact with IL-12, which differentiates them into Th_1 phenotypes (Chapter 3). Th_1 phenotypes will interact with B cells through MHC Class I TCRs and activate them (Chapter 3). IL-12 is also secreted by Th_{17} T lymphocytes to induce IL-17/IFN-γ producing T cells

(Chapter 3). Furthermore, IL-12 is secreted by mature DCs to induce NK cells. IL-12 is a potent pro-inflammatory inducer, and is represented in the cytokine milieu without semen 1.59-fold over mock. However, there is little increase beyond this concentration when semen is apically exposed to any of the three cell lines (Chapter 5).

MIP-1β, as another cytokine of interest, goes from an average across all three cell lines of just above 2.0-fold change over wells that are only co-cultured with PBMCs to a little less of an average fold-change of 1.58 over PBMC wells only (Chapter 5). MIP-1β can activate granulocytes, which are found throughout the FRT to attenuate immune cell chemotaxis to the site of inflammation and in doing so also induce macrophages to produce IL-6 and TNF-α [102-104]. The increases seen in the supernatant do not seem to reflect this happening as IL-6 and TNF-α were both an average of less than 0.55-fold change over PBMC only wells (Chapter 5). What does stand out about MIP-1β and RANTES, however, is that both of these are CCR5 ligands [135, 137, 168, 169].

6.6 The effect of semen across the female reproductive tract epithelia has broader implications for pathogen transmission

One of the most prevalent contexts for looking at how semen modulates the FRT is pathogen transmission. In the case of human immunodeficiency virus type 1 (HIV-1) transmission, the increases in MIP-1β and RANTES could have implications with transmission, because CCR5 is one co-receptor the virus utilizes

to enter and infect target cells. However, the other effects of semen would have implications for other pathogen transmission.

When semen increases tight junction formation and decreases permeability across the epithelium, any pathogens found in semen would not be able to move between cells across the epithelial barrier to establish infection in the subepithelial space [104, 121, 126, 170] (Appendix Chapter 2). The pathogens would have to traverse the barrier intracellularly, which poses a much greater challenge to pathogen. This effect is also going to be region specific as was seen with region specific changes in TEER due to semen (Appendix Chapter 2). END1 cells had a significant, sharp increase in TEER in as little as 5 minutes, which lasted over 4 h (Appendix Chapter 2). The other cell lines tested did not see this increase so quickly. Although characterized by a single, columnar epithelium, END1 cells may not provide a leakier barrier as compared to the squamous, stratified epithelia of VK2 or ECT1 cells (Appendix Chapter 2).

However, the effect of semen across the different regions of the FRT may have broader implications for pathogen transmission requiring the use of immune cell hosts, such as HIV-1 [95, 97, 104, 126, 171, 172]. As stated above, CCR5 is one of the co-receptors needed for entry into target $CD4^+$ T cells (T helper cells) in conjunction with the CD4 receptor. CXCR4 is another co-receptor that can be used in conjunction with CD4, by HIV-1, to enter and infect T helper cells. If an HIV-1 virion utilizes CCR5 and CD4 it is termed R5, and if an HIV-1 virion utilizes CXCR4 and CD4 it is termed X4 [108, 131, 139].

Viral tropism is an important consideration in the establishment of the initial infection [108, 132]. While both virus co-receptor phenotypes may be present in semen at the time of deposition in the FRT, in discordant, heterosexual transmission of HIV-1 R5 viruses are almost always the HIV-1 variants to enter and infect T helper cells that drain to the lymph nodes and establish a chronic infection [132]. While X4 viruses can be transmitted, the transmission of viruses that use CXCR4 as a co-receptor is rare [132]. In studies of semen and its ability to transduce its effect across the epithelial barrier and influence immune cells, it was found that the PBMCs co-cultured with Ect1 epithelial cells, which represents the transformation zone, were preferentially infected with R5 viruses than X4 viruses in the presence of semen (Chapters 4 and 5). However, what was interesting about this study is that in the absence of semen, X4 viruses were the most abundant HIV-1 variant to enter and infect T helper cells (Chapters 4 and 5). Therefore, the presence of semen in the FRT releases some factor from the epithelium that acts on immune cells present in the subepithelial space to increase the chances of R5 viral entry and infection of X4 entry and infection (Chapters 4 and 5).

Aiding in the increased infection of R5 viruses in the presence of semen is the increased surface density of CCR5 (Chapter 5). When comparing the surface density of CCR5 on each regions' T helper cells the ECT1 cell line induced a significantly higher density of CCR5 than the VK2 or END1 (Chapter 5). The cytokine milieu in the subepithelium of the ECT1 cells appears to favor CCR5 upregulation (Chapter 5). However, the cytokine environment induced by ECT1

cells is the strongest pro-inflammatory signal of all three cell lines (Appendix Chapter 1), and a more pro-inflammatory environment will increase the surface density of CCR5 on T cells [139, 147, 153].

When looking at CXCR4 surface density across all cell lines, we see the opposite (Chapter 5), insofar T helper cells co-cultured with ECT1 cells had the lowest surface density of CXCR4 compared to the other cell lines (Chapter 5). This fact might be one reason why T helper cells co-cultured with VK2 and END1 cell lines were largely infected with X4 viruses over R5 viruses in the absence and presence of semen (Chapter 5). This data together suggest that in the presence of semen there are factors released from the ECT1 cell line (and ectocervix, by extension) that are specific to that region that enhance CCR5 surface density and infection of R5 viruses (Chapters 4 and 5).

6.7 Future Directions

The dynamic interplay of semen and the FRT has direct implications in reproductive biology but can also contribute to pathogen transmission. As seen in this review constituents in semen interact with the epithelial barrier to induce mechanical changes to occlude paracellular pathogen transmission. Identifying the specific factors in semen that work together to rapidly induce tight junction regulation as early as 5 minutes-post semen exposure could lead to the development of powerful microbicides. The act of coitus is known to induce microabrasions in the FRT [127], therefore by developing a topical agent that

would help reduce the amount of paracellular permeability may offer an approach that reduces the risk of pathogen transmission from male-to-female.

Not just with mechanical processes but by understanding what factors in semen contribute to the overall success of pregnancy and fetal development new conclusions can be made about the causes of infertility. Knowing what factors cause infertility and how they have long term effects could change how infertility is treated. However, those same immunomodulatory factors in semen have undesired consequences in pathogen transmission. As stated above, viral transmission of HIV-1 (either by R5 or X4 viruses) is enhanced in the presence of semen (Chapters 4 and 5). Therefore, uncovering what specific factor is released from the epithelium to enhance transmission would be important. This unknown factor could be an ideal target for microbicide development. Identifying the specific factor that enhances R5 viral transmission in the transformation zone will provide clues and insight to how viruses find, enter, and infect cells.

6.8 Conclusion

It is essential to understand the importance of semen and the downstream effects it may have in areas like the FRT. Semen is far from a chemically inactive substance that just delivers paternal antigens to the female to establish pregnancy. This review has shown that semen elicits a mechanical and biological effect on the FRT epithelia. Therefore, the need to understand how these effects take place is paramount to not only the safety and well-being of the female but the safety and well-being of, perhaps, a developing fetus. There are far more studies that need to

be done to elucidate the full effects of semen and how it changes the FRT relative to conception and pathogen transmission.

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Appendix Chapter 1

Semen induces polarized time- and tissue-dependent release of immunomodulatory factors from human cervicovaginal epithelial cell lines

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A1.1 Abstract

Semen is a biologically complex fluid containing numerous immunomodulatory factors. Previous studies concerning the effects of semen on epithelial cell soluble factor secretion have relied on the use of submerged cell culture methods, which do not accurately reflect the polarized nature of the epithelium lining the female reproductive tract. To circumvent this limitation, a transwell tissue culture system was used to examine the effects of semen on polarized cervicovaginal epithelial cells. Cell lines derived from different regions of the reproductive tract – vagina, ectocervix, and endocervix – were grown into polarized, confluent monolayers and then exposed to 25% seminal fluid for 4 h. Quantification of analytes contained in conditioned media collected at 4 h and 24 h post-exposure demonstrated time-, cell line-, and polarity-dependent cytokine, chemokine, and growth factor secretion in response to semen, with vaginal and endocervical cells being the most and least responsive, respectively. Differences in semen-induced factor release between different regions of the female reproductive tract may alter immune responses to pathogens within the FRT as well as modulate the risk of infection.

A1.2 Introduction

Traditionally, studies of both reproduction and sexually transmitted infections have analyzed microbial and immunological events independent of seminal fluid, which has generally been viewed as an inert vehicle for the delivery of sperm and sexually transmitted disease (STD) pathogens. However, recent evidence indicates that seminal fluid is more than a passive carrier and is actually complex in composition, containing many factors, including chemokines, cytokines, and growth factors. This work has primarily stemmed from research on infertility, which seeks to understand the role that factors in semen may play in facilitating successful reproduction. These studies have revealed that seminal fluid is instrumental in driving the local immune changes within the female reproductive tract (FRT) that are integral to fertilization as well as embryo implantation.

Although semen induces an initial local inflammatory response due to recognition of foreign paternal antigens by the female, there is an eventual decline in inflammation and a state of immune tolerance that prevents immune clearance of sperm and the ensuing embryo allograft [87]. The shift from inflammation to tolerance is orchestrated not only by factors contained in seminal fluid, but also by another major participant: the epithelial monolayer lining the FRT. Seminal fluid can act directly on the epithelium by altering the function of the epithelial barrier [29], and indirectly by inducing the secretion of factors that will, in turn, direct local immune responses. Together, semen-derived factors and epithelial-derived factors direct the biphasic immune response necessary for sperm clearance and, eventually, tolerance.

The same factors that are critical to fertilization may also affect the susceptibility of women to infection by STD pathogens, including the human immunodeficiency virus type 1 (HIV-1). Despite effective antiretroviral therapies, the HIV-1 epidemic continues to be a problem, with over 2.5 million new infections in 2011 [173, 174]. About half of the world's HIV-1-infected individuals are women, the majority of whom acquired the infection through male-to-female sexual transmission [2]. Females are twice as likely as men to become infected during intercourse, with a 1-in-2000 risk of a woman acquiring HIV-1 from her infected male partner, as compared to the 1-in-7000 risk for a male becoming infected by a HIV-1-infected female [2]. In order to better understand the factors that put women at risk for infection in the current epidemic, it will be essential to closely study the initial steps of HIV-1 transmission that occur within the FRT.

For this purpose, we turned to an *in vitro* cell culture system that more accurately mimics the FRT. Previous semen-epithelium interaction studies have relied on traditional submerged cultures in which cells are grown in flat-bottom plates. Although the cells grow rapidly in this setting, the polarized aspect of the cell monolayer is not properly reproduced and only one interface (apical) is available to the external environment. More recent studies have relied on a transwell culture system [8, 148] in which cells are grown on semi-permeable transwell membrane inserts and monitored for confluence to ensure the development of fully intact monolayers. Transwell-based studies of cell lines derived from different regions of the FRT have identified differences in tight junction expression and phenotype unique to each region, which is consistent with

analyses of *ex vivo* tissues isolated from these areas [175]. Importantly, stimuli in the transwell system can be applied as they may be encountered *in vivo*; in our model, the apical chamber represents the FRT lumen where semen is deposited during intercourse, and the basolateral chamber represents the subepithelial space.

This method of culture also permits us to monitor the dual functions of epithelial monolayer: the “fence” and the “gate” [176]. Tight junction strands, expressed in the apical region of the membrane, are responsible for connecting the neighboring cells to form the “fence” network, resulting in cell polarity and structure [176]. At the same time, tight junctions also function as a “gate” to regulate paracellular permeability, blocking larger entities, such as bacterial pathogens or cancer cells; maintaining osmotic balance; and regulating the passage of molecules and ions [176]. These functions have been well characterized in many tissues using measurements of electrical resistance and permeability, and have also been reviewed in the literature [157, 175-178]. Therefore, in our system, the transwell culture system provides an excellent platform for examining the effects of semen on the female epithelium at the level of both barrier integrity [29] and the polarized release of soluble factors.

We hypothesized that epithelial cells grown in a transwell culture system would respond to semen by releasing factors in a polarized manner – a key aspect that cannot be studied in a submerged culture. In order to determine the identities of biologically active components released by the epithelium, we exposed epithelial monolayers to seminal fluid (SF) as they would be encountered *in vivo* during

coitus and analyzed the supernatants to determine how factors in SF contributed to the initial inflammatory response by the epithelium. The goal of these investigations was to study the early steps of heterosexual transmission *in vitro* using a quick, effective, and inexpensive assay that can be used to show changes in epithelial function and their potential effects of HIV-1 transmission.

A1.3 Materials and Methods

A1.3.1. Cell line maintenance

Experiments were performed using cell lines derived from three regions of the human female reproductive tract: endocervical cells (End1/E6E7), ectocervical cells (Ect1/E6E7), and vaginal keratinocytes (VK2/E6E7) (ATCC, Manassas, VA). Prior to seeding, cells were grown and maintained in T150 tissue culture flasks (Grenier Bio One, Monroe, NC) at 37°C and 5% CO_2. Ect1 cells were cultured using the Keratinocyte Serum-Free Medium (Gibco, Grand Island, NY), which included 50 µg/ml bovine pituitary extract (BPE), 0.1 ng/ml epithelial growth factor (EGF), and 50X penicillin/streptomycin solution. End1 and VK2 cells were cultured in the same Keratinocyte-SFM medium described above but with the addition of 0.4 mM calcium chloride (EMD Millipore, Billerica, MA).

A1.3.2. Transwell culture system

Cell lines were cultured as confluent, polarized monolayers by seeding into the apical chamber of a 6.5 mm, 0.4 µm pore size, polyester (PET) transwell insert in a 24-well receiver plate (Corning, Corning, NY). All cell lines were seeded at a density of 1.5×10^5 cells per well in 200 µl of media in the apical chamber and 600

µl in the basolateral chamber. Time to confluence (determined by electrical resistance, as described below) was cell line-dependent and varied between 14 and 21 days post-seeding.

A1.3.3. Transepithelial electrical resistance

Epithelial monolayer establishment was monitored by measuring transepithelial electrical resistance (TEER or TER), which is an indirect measure of tight junctions. Using an EVOM2 voltohmeter and STX2 chopstick electrode (World Precision Instruments, Sarasota, FL), daily resistance readings (ohms or Ω) were taken during the growth of the monolayers. Cell monolayers were determined to be fully developed once the TEER readings had plateaued for 2 to 3 consecutive days. Empty wells containing media only (no cells) were used to establish background TEER levels. Due to differences in the expression of tight junctions, raw plateau TEER values (Ω) differed between cell lines: End1 cells, ~250-300 Ω; Ect1 cells, ~220-250 Ω; and VK2 cells, ~220 Ω. These results are consistent with other studies using these cell lines [148, 175].

2A1.3.4. Seminal fluid

Seminal fluid (SF) was purchased as 10 ml of pooled semen from a mixture of six healthy donors between 26 and 34 years of age (Lee Biosolutions, St. Louis, MO). Specimens were shipped overnight on dry ice and immediately divided into 500 µl aliquots and frozen at -20°C upon arrival. In all descriptions, SF refers to whole semen containing sperm.

A1.3.5. Collection of conditioned media

End1, Ect1, and VK2 monolayers in transwells were exposed apically to 25% SF in duplicate for 4 h, and then apical and basolateral supernatants were collected and stored at -80°C. The monolayers were washed two times with HBSS and fresh media was added to both chambers. After an additional 20 h incubation, the apical and basolateral supernatants were again collected and stored at -80°C. For experiments involving HIV-1, the monolayers were exposed apically to HIV-1 laboratory strain BaL (Zeptometrix, Buffalo, NY) in the absence or presence of 25% SF for 4 h, followed by the collection of apical and basolateral supernatants. All conditioned media samples were analyzed by Luminex analysis.

A1.3.6. Luminex assay of conditioned media

The 30-plex assay was performed as described by the manufacturer (Millipore, Billerica, MA). Briefly, each well of the 96-well filter plate was pre-equilibrated using assay buffer prior to the addition of the conditioned media (apical or basolateral) samples. The cytokine standards and samples were diluted to 2X in assay buffer and added in duplicate to the designated well of the filter plate, then incubated with antibody-coupled beads with shaking for 1 h and without shaking for 1 h. Next, secondary antibody was added to the wells and incubated with shaking for 30 min and without shaking for 30 min. Unbound analytes were removed through the well filters using a vacuum manifold. After washing the wells, the samples were then analyzed on a Luminex200 xMap plate reader (Applied Biosystems, Carlsbad, CA, USA) [179, 180]. Each sample was assayed for 30 different factors, including cytokines and chemokines, and graphed using Excel.

The benefit of using Luminex technology is the sensitivity of the assay and ability to detect cytokine proteins in small sample volumes. Semen samples were also analyzed using the 30-plex Luminex assay.

A1.3.7. Statistical analyses

p-Values were calculated for the difference between pairs of treatments conditions using a two-tailed Student's *t*-test, and a value of less than 0.05 was set as the limit for statistical significance. p-Values are indicated on the figures and within the figure legends.

A1.4 Results

A1.4.1. Epithelial monolayer viability and monolayer integrity are unaffected by SF exposure

Three well characterized cell lines were used to represent the distinct regions of the FRT. End1, Ect1, and VK2 cell lines express variable levels of tight junctions, which correlate with their respective functions in the regions from which they were derived [83, 175]. It is important to mention that each cell line has a transwell TEER reading at confluence that corresponds to the level of tight junction expression *in vivo*: VK2 < Ect1 < End1 [175]. VK2 cells (vaginal keratinocytes) were derived from cells that form an effective mechanical barrier that is up to 25 squamous cell layers thick, but have low expression of tight junctions facilitating the continual sloughing of the lining. Ect1 cells (ectocervical cells) represent the transition zone, an area in which the thick squamous epithelium begins to thin and gains a columnar phenotype that expresses high levels of tight junctions. End1

cells (endocervical cells) form single, organized layers of columnar epithelium that express high levels of tight junctions [175]. Previous studies of a similar nature were performed by culturing these cell lines in flat-bottom plates rather than transwells [29, 84].

Several studies have reported decreased cellular viability in the presence of SF, resulting in limitations with respect to exposure time and usable concentration ranges [7, 84]. While dilution of semen to concentrations as low as 0.1% can circumvent these limitations, these concentrations are too low to fully replicate the concentrations of semen that occur after deposition in the FRT. Studies of human and animal reproduction have revealed that once in the FRT, SF is diluted by mixing with vaginal secretions and spreading throughout the vaginal tract [114]. Although there is some disagreement about the actual degree of dilution that occurs, the general consensus is that semen is diluted to between 25% and 10% of its original concentration [114]. With respect to semen retention in the FRT, observations on the absorption, spreading, and breakdown of SF that occurs within the FRT have determined that the majority of SF has been removed or exited the region by 4 h [114]. These factors are important to define in order to develop an *in vitro* approach that better mimics *in vivo* conditions and events. We speculated that past studies have not been able to achieve physiological concentrations due to the hypersensitivity of the cells to semen in submerged culture. Using this information to guide our experimental design, End1 monolayers were incubated apically with 25% or 10% SF for 4 h. In this time frame, SF exposure had no effect on cell viability (data not shown). Similar results were

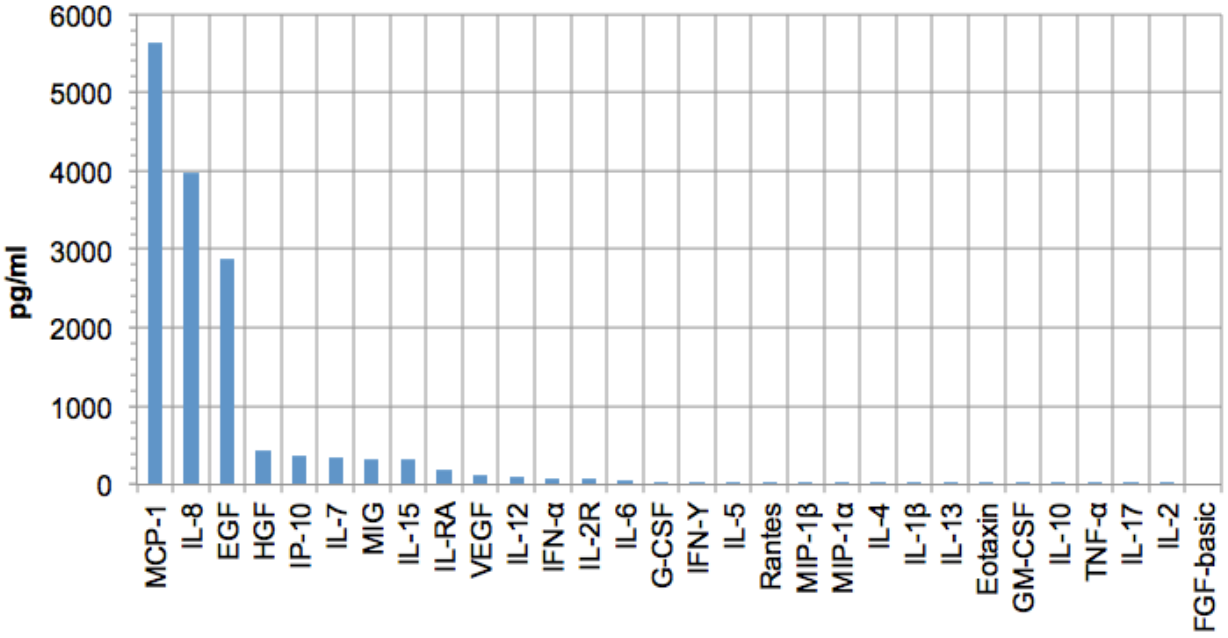
observed when the experiment was repeated using Ect1 or VK2 monolayers (data not shown). With respect to the integrity of the monolayer, we have shown that the apical application of 25% SF to these cells in transwell cultures caused a rapid and significant increase in TEER [29], reinforcing the conclusion that cell line monolayers (i) remain viable after SF exposure and (ii) serve to effectively partition the semen in the upper chamber from the media in the lower chamber.

A1.4.2. Seminal fluid has a proinflammatory profile and contains a multitude of immunomodulatory factors

Before defining the response of the epithelial monolayer, the contents of the semen were profiled in order to determine what factors would be introduced into the apical transwell environment during SF exposure. Since SF is highly variable not only between donors, but even between ejaculates collected from the same individual on different days, pooled specimens from multiple donors were utilized in order to reduce the variability and obtain a more representative picture of semen factor content across an average population [120]. While SF was shown to be rich in a wide array of factors, the most prominent among the 30 factors analyzed were chemokines (Fig. A1.1). In agreement with other published studies, our analyses indicated that semen from the average healthy male is generally characterized as slightly pro-inflammatory, as detectible concentrations of inflammatory factors such as IL-12, IFN-α, IL-6, and IFN-γ were present (Fig. A1.1B) [6, 7]. In contrast, anti-inflammatory cytokines were almost undetectable, with the exception of IL-1RA (177 pg/ml) (Fig. A1.1B). There were also high concentrations of chemokines and growth factors present, which have been widely discussed in literature for their

pivotal biological role in reproduction and embryogenesis [87, 114]. The most prominent chemokines in pooled SF were monocyte chemotactic protein 1 (MCP-1) (5637.5 pg/ml) and interleukin 8 (IL-8) (3971.4 pg/ml) (Fig. A1.1C), while the most concentrated growth/signaling molecule was epithelial growth factor (EGF) (2867.1 pg/ml) (Fig. A1.1D).

Figure A1.1A



MCP-1	5637.5	MIG	325	IL-2R	69.02	MIP-1β	14.24	GM-CSF	2.48
IL-8	3971.40	IL-15	322.48	IL-6	58.08	MIP-1α	10.78	IL-10	2.18
EGF	2867.1	IL-RA	177.71	G-CSF	30.67	IL-4	6.37	TNF-α	1.25
HGF	437.40	VEGF	130.72	IFN-γ	26.12	IL-1β	5.94	IL-17	0.87
IP-10	372.79	IL-12	107.49	IL-5	26.08	IL-13	5.62	IL-2	0.48
IL-7	341.43	IFN-α	81.83	Rantes	15.96	Eotaxin	5.018	FGF-basic	0

Figure A1.1B

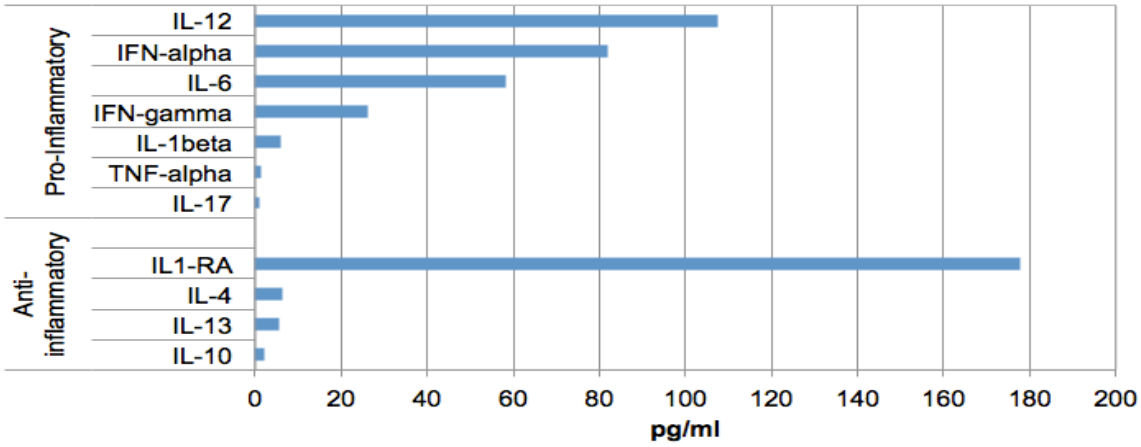

Pro-Inflammatory
IL-12
IFN-alpha
IL-6
IFN-gamma
IL-1beta
TNF-alpha
IL-17
Anti-inflammatory
IL1-RA
IL-4
IL-13
IL-10
0
20
40
60
80
100
120
140
160
180
200
pg/ml

Figure A1.1C

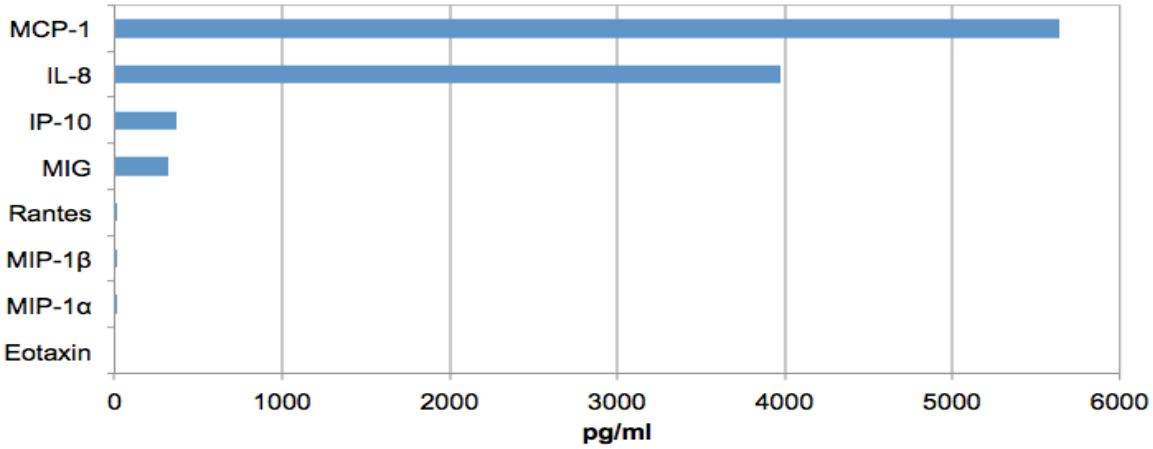

MCP-1
IL-8
IP-10
MIG
Rantes
MIP-1β
MIP-1α
Eotaxin
0
1000
2000
3000
4000
5000
6000
pg/ml

Figure A1.1D

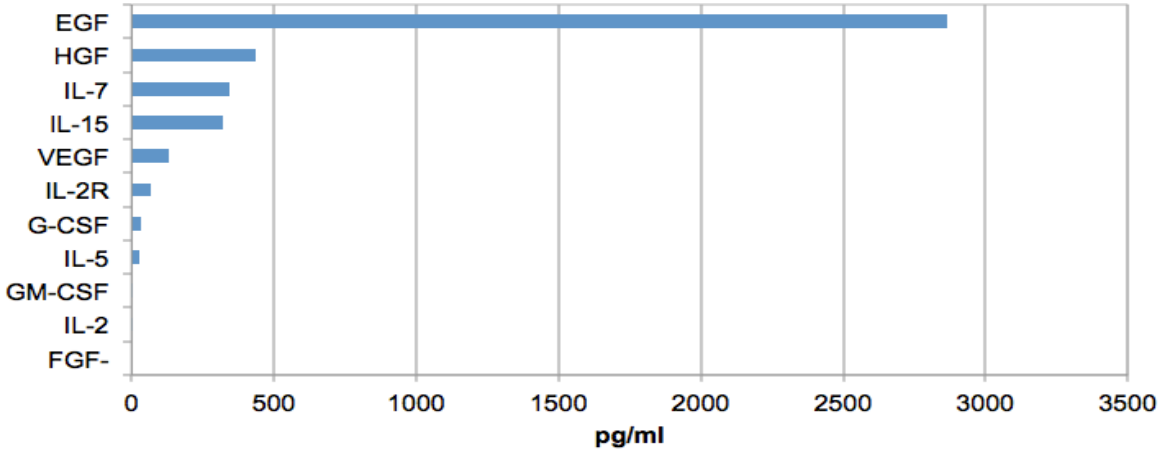


Fig. A1.1. Concentrations of biological factors contained in pooled human seminal fluid. (**A**) Analytes detected by Luminex assay in 25% diluted SF were arranged from highest to lowest concentration (pg/ml). The table shows the exact concentrations detected (pg/ml). (**B-D**) The factors are arranged according to different biological activities: (**B**) pro-inflammatory and anti-inflammatory cytokines, (**C**) chemokines, and (**D**) growth/signaling factors. Analytes are arranged from highest-to-lowest concentration (pg/ml).

A1.4.3. Polarized release of epithelial factors during SF exposure

After demonstrating that the content of SF was predominantly inflammatory, the next step was to characterize the influence of semen on the epithelium in the context of the vaginal lumen during intercourse. We have characterized the direct effects of SF on the epithelium at the level of barrier integrity (Appendix Chapter 2), but here we were interested in measuring the indirect effects of exposure by identifying the secretion of factors from the epithelium as a response to semen. A previous study on the effects of seminal plasma on genital epithelial cells (GECs) in transwell inserts demonstrated that these cells unilaterally released cytokines exclusively into the apical chamber, while the same factors were undetectable in the basolateral chamber [8]. In our model, all three cell lines secreted factors at basal levels into both the apical and basolateral chambers of the mock-exposed wells, and levels of release were generally increased after exposure to SF (Fig. A1.2). Overall, the profile of factors secreted into both chambers by all three SF-exposed, FRT-derived cell lines at both time points was predominately pro-inflammatory (Table A1.1). The only anti-inflammatory factor released at a level greater than 5-fold above its basal level was IL-1RA. However, IL-1RA returned to mock-exposed levels by 24 h post-exposure (Table A1.1). Heat maps show fold changes in factor release into the apical (Fig. A1.3) and basolateral (Fig. A1.4) conditioned media from all three cell lines.

When comparing the 4 and 24 h supernatants, it was apparent that there was a time-dependent aspect of the effects of SF on some factors. The basolateral

Figure A1.2

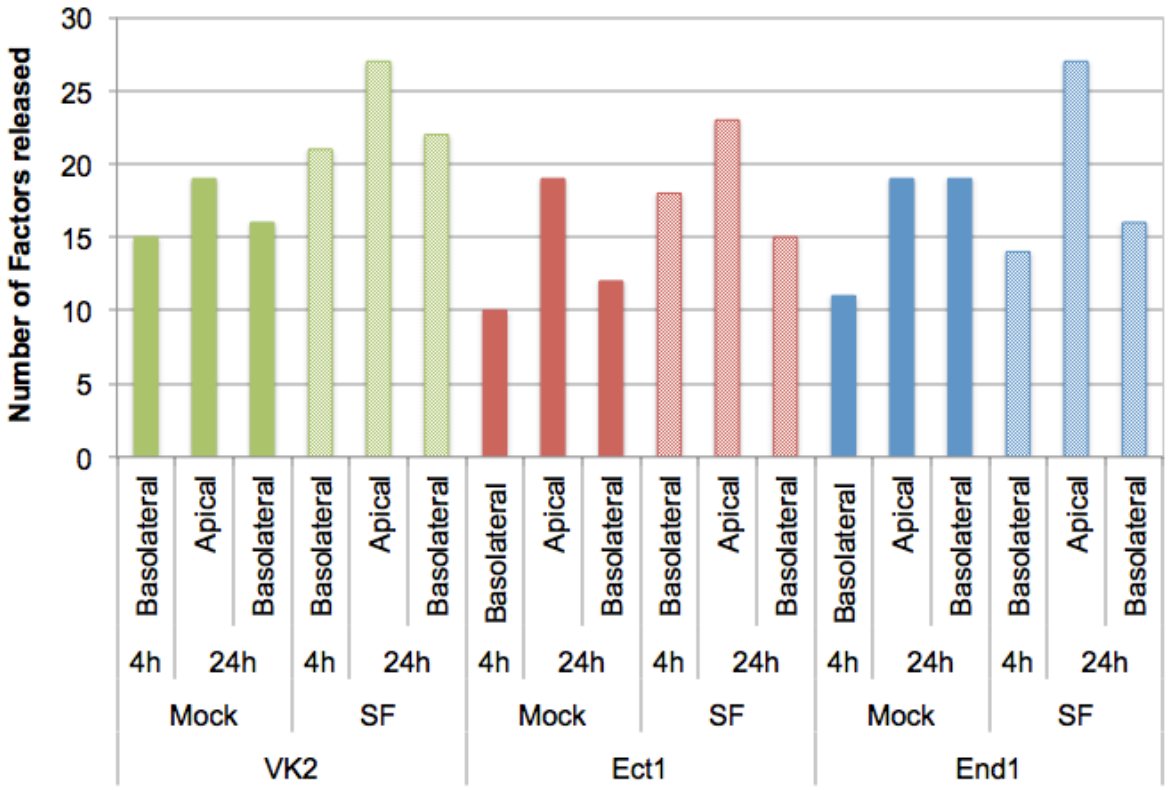


Fig. A1.2. Immunomodulatory factor release from FRT epithelial cells is time-, direction-, and cell line-dependent. Conditioned media samples were collected at 4 and 24 h post-exposure from cells exposed for 4 h to semen. The numbers of factors secreted out of the 30 analytes are plotted with respect to chamber of origin, time post-exposure, the absence (mock) or presence of SF, and the cell type.

Figure A1.3

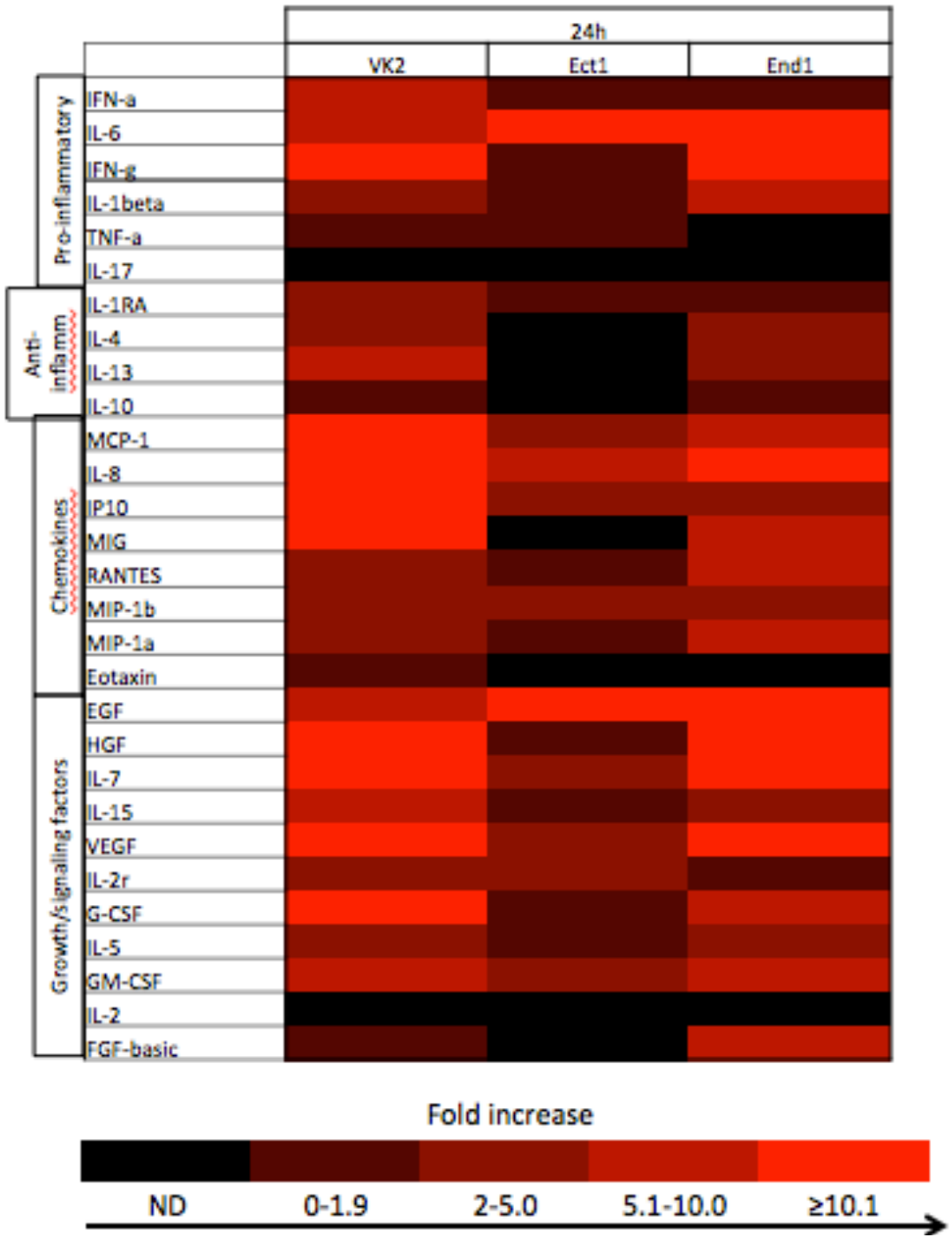

24h
VK2
Ect1
End1
Pro-inflammatory
IFN-a
IL-6
IFN-g
IL-1beta
TNF-a
IL-17
Anti-inflamm
IL-1RA
IL-4
IL-13
IL-10
Chemokines
MCP-1
IL-8
IP10
MIG
RANTES
MIP-1b
MIP-1a
Eotaxin
Growth/signaling factors
EGF
HGF
IL-7
IL-15
VEGF
IL-2r
G-CSF
IL-5
GM-CSF
IL-2
FGF-basic
Fold increase
ND
0-1.9
2-5.0
5.1-10.0
≥10.1

Fig. A1.3. Relative changes in apical factor release are time-, direction-, and cell line-dependent. Changes in factor concentrations in the apical chambers are shown as a heat map of fold-change over mock of 30 analytes detected in conditioned media collected from the apical supernatants at 24 h after exposure to 25% SF.

Figure A1.4

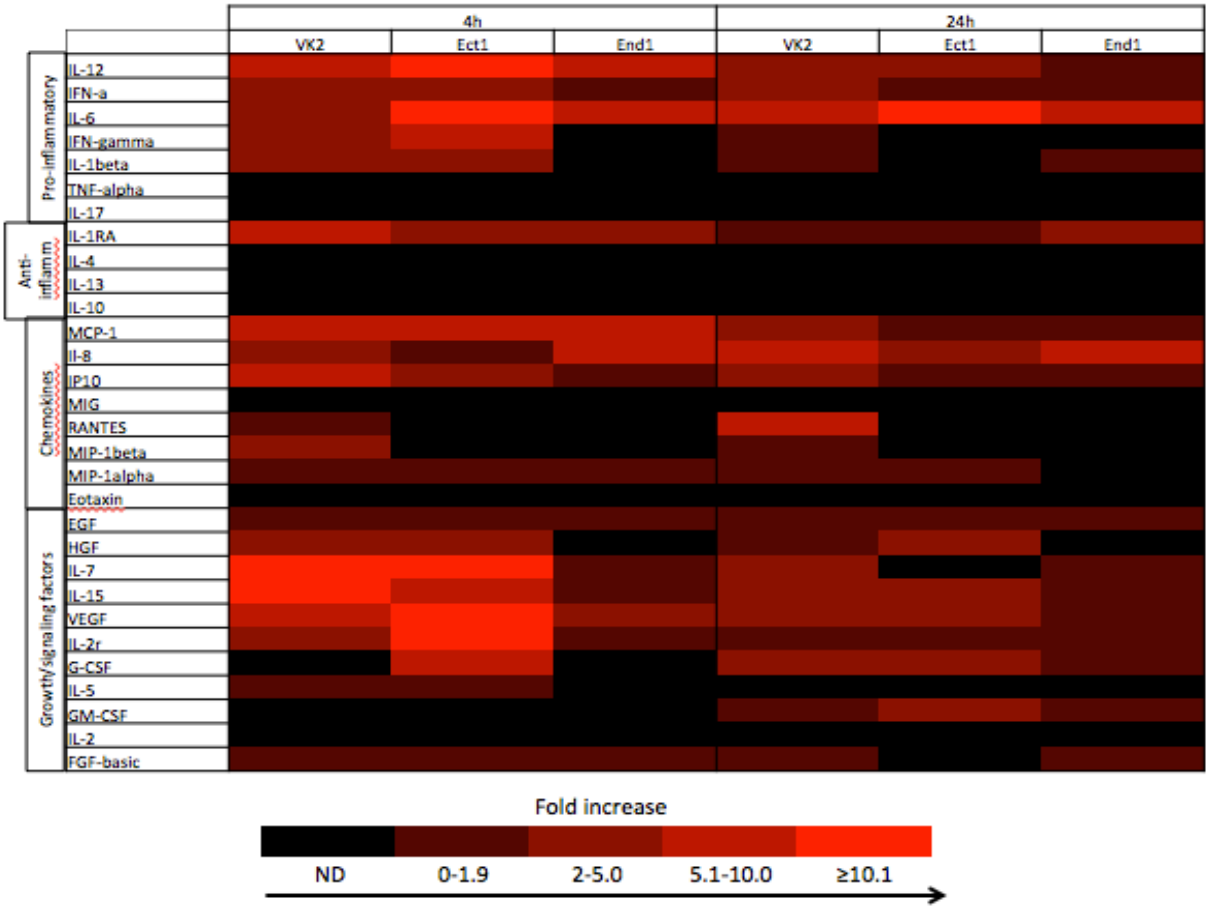


Fig. A1.4. Relative changes in basolateral factor release are time-, direction-, and cell line-dependent. Changes in factor concentrations in the basolateral chambers are shown as a heat map of fold-change over mock of 30 analytes detected in conditioned media collected from the basolateral supernatants at 4 and 24 h after exposure to 25% SF.

Table A1.1

Table A1.1. Factor release as a consequence of SF exposure. Fold change of each analyte in the conditioned media collected from the apical and basolateral chambers at both time points across all three cell lines after exposure to 25% SF. BD, below the assay limit of detection.

		VK2		Ect1		End1	
		4h	24h	4h	24h	4h	24h
IL-1β	Apical	20.5	3.2	18.6	1.0	36.4	5.3
	Basolateral	3.3	1.8	2.0	BD	BD	0.3
IL-1RA		10.2	2.2	1.9	0.6	5.5	1.8
		5.6	1.1	2.6	1.8	3.7	2.8
IL-2		1.2	BD	BD	BD	BD	BD
		BD	BD	BD	BD	BD	BD
IL-2R		5.7	3.8	1.8	2.4	4.7	1.7
		3.7	1.2	25.0	1.2	1.0	1.3
IL-4		14.7	4.0	11.9	BD	15.0	3.0
		BD	BD	BD	BD	BD	BD
IL-5		23.0	4.3	12.9	0.6	29.4	2.7
		0.6	BD	0.6	BD	BD	BD
IL-6		25.7	9.9	35.1	15.2	23.8	11
		5.0	5.1	14.5	24.3	9.7	5.1
IL-7		130.6	12.9	110.2	2.7	416.6	36.5
		25.1	2.7	44.0	BD	1.3	0.8
IL-8		73.0	29.6	12.6	5.2	71.3	11.6
		3.4	5.6	1.8	3.3	6.0	9.1
IL-10		4.1	1.3	2.9	BD	4.1	1.2
		BD	BD	BD	BD	BD	BD
IL-12		73.1	8.0	270.8	1.4	64.8	8.7
		6.5	2.1	31.3	2.5	5.9	1.3
IL-13		15.7	6.8	14.4	1.0	14.9	4.0

		BD	BD	BD	BD	BD	BD
IL-15		55.7	8.2	71.9	1.2	35.6	2.7
		22.0	2.9	7.7	2.3	1.4	1.0
IL-17		1.6	BD	BD	BD	BD	BD
		BD	BD	BD	BD	BD	BD
FGF-basic		1.4	1.2	BD	BD	0.9	5.4
		0.9	0.7	1.6	BD	1.7	1.3
MIG		BD	15.6	1004.3	BD	1358.6	6.0
		BD	BD	BD	BD	BD	BD
MCP-1		1182.6	16.4	97.6	2.1	930.4	7.2
		7.4	3.3	5.9	1.3	6.5	1.8
MIP-1α		22.3	3.4	27.6	0.5	18.8	6.7
		1.0	0.7	1.4	1.0	1.4	BD
MIP-1β		21.6	2.4	28.0	3.0	17.8	4.6
		2.2	0.6	BD	BD	BD	BD
IFN-γ		72.6	15.5	40.5	1.6	74.9	16.7
		3.6	1.3	5.9	BD	BD	BD
IFN-α		22.0	5.1	13.3	1.3	11.7	1.7
		2.1	2.2	2.4	1.5	1.7	0.7
TNF-α		3.3	0.9	3.6	0.7	2.9	BD
		BD	BD	BD	BD	BD	BD
EGF		4061.1	7.4	1445.8	12.5	3148.8	16.7
		1.5	1.0	1.3	1.1	1.2	1.5
G-CSF		25.6	10.8	70.7	1.3	73.2	6.2
		BD	3.5	6.9	3.5	BD	1.0
GM-CSF		4.8	7.3	1.5	4.5	5.6	9.1
		BD	1.2	BD	2.3	BD	1.3
HGF		256.1	13.7	155.3	1.9	264.7	13.7
		2.1	1.1	2.1	2.1	BD	BD
VEGF		303.0	39.4	418.2	2.6	265.0	36.4
		9.3	2.5	1.0	2.5	3.9	1.9
IP-10		148.2	11.2	121.8	2.4	28.4	2.1
		6.6	3.1	4.6	1.4	1.6	1.2

Eotaxin		3.6	0.1	1.7	BD	3.6	BD
		BD	BD	BD	BD	BD	BD
RANTES		5.9	2.7	18.7	1.2	23.5	9.7
		0.9	5.2	BD	BD	BD	BD

compartment at 4 h included an overall increase in secretion activity that continued out to 24 h, which was also true for the apical compartment at 24 h (Fig. A1.2). When the fold change in factor secretion was compared between individual analytes, it was apparent that some were secreted at a maximum concentration early (4 h post-exposure) and decreased to nearly mock-exposed levels by 24 h. For example, IL-7 from VK2 and Ect1 cells peaked early (4 h post-exposure) at 25- and 44-fold above mock-exposed levels, respectively (Table A1.1). On the other hand, some factors continued to accumulate, such as VEGF, which increased nearly 40-fold by 24 h in the apical chamber of VK2 cells (Fig. A1.3 and Table A1.1), and IL-17, which increased 3.5-fold in the basolateral chamber (Fig. A1.4 and Table A1.1). However, not all factors that were present in the conditioned media at 24 h were expressed at the early time point; IL-17 and RANTES were detected only in the 24 h apical and basolateral supernatants of VK2 cells (Table A1.1).

In this experiment, polarized release of factors was also noted. While IL-6 was consistently secreted by all three cells lines into both chambers at each time point (Figs. 3 and 4, and Table A1.1) some factors were exclusively released either into the apical or basolateral chamber. For example, IL-2R release by Ect1 cells at the 4 h time point was increased 25-fold in the basolateral chamber, but present at less than 2-fold in the apical chamber relative to mock (Table A1.1). In the same cells, IL-5 release was limited to the apical supernatant (~13-fold) and remained undetectable in the basolateral chamber (Table A1.1). The disparate concentration of IL-5 across the monolayer interface also provides evidence for efficient tight

junction interactions, proving that the seal regulating the paracellular space is tight enough to prevent factors secreted in great abundance in the apical chamber from diffusing into the bottom chamber.

The integrity of the epithelial monolayer as a barrier to diffusion was also indicated by differences in concentrations between factors present in SF in the apical chamber and the same factors detected in the basolateral chamber. IFN-γ was present at low concentrations in SF (26.12 pg/ml) (Table A1.1). In contrast, VK2 cells subsequent to SF exposure released 72.5 pg/ml apically at the 4 h time point, which was still elevated to ~26 pg/ml at 24 h post-exposure despite the removal of SF; the same relationship was observed with IFN-γ in the End1 cells (Table A1.1). On the other hand, MCP-1, which was present in semen at very high concentrations – over 5500 pg/ml – was released into the basolateral chamber by all three cell lines at very low concentrations (~30 pg/ml or less) (Table A1.1). This is a key observation, indicating that factors in the basolateral chamber were released from the epithelial cells rather than diffusing out of the semen across the epithelial barrier. Further study will be required to determine whether significant correlations exist between the concentrations of factors found in seminal fluid and the concentrations released by the epithelium as a response. Establishing relationships between these variables will aid future investigations focused on the effects seminal factors on infertility and STD pathogen transmission within the FRT.

The response of the epithelium to semen varied across the different regions of the FRT, as represented by the three FRT-derived cell lines. Cells from the

vaginal tract were the most active, secreting the widest range of factors in both the absence and presence of semen at both time points compared to the other two cell lines (Figs. 2-4 and Table A1.1). However, with respect to fold change, the ectocervical cells had the greatest response to SF at 4 and 24 h in the basolateral chamber, releasing high concentrations of IL-2R, IL-6, IL-7, and IL-12 (Fig. A1.4 and Table A1.1). Endocervical cells were the least responsive to semen (Table A1.1), producing a few factors at 2-fold or more levels at either time point (including IL-8, IL-6, VEGF, and MCP-1). Some cytokines were exclusively expressed by one cell line. For example, FGF-basic, which was not detected in semen (Fig. A1.1), was not secreted by VK2 or Ect1 cells, but was increased 5.4-fold in the apical chamber of End1 cells at 24 h (Table A1.1). In summary, these analyses indicate that the epithelial response to SF, which continues through 24 h post-exposure even in the absence of SF, is directional, predominantly pro-inflammatory, and dependent on the identity of the epithelial cells.

A1.4.4. Effects of HIV-1 BaL on epithelial cytokine release

Previous studies have shown variable effects of HIV-1 on the epithelial barrier. Although epithelial cells may or may not host HIV-1 infection, they are still positioned to provide an innate immunological response to cell-free (or cell-associated) virus. The release of factors by the epithelium will shape the local immune response and could possibly affect the early events of HIV-1 transmission. To examine this effect, Ect1 epithelial cell monolayers were exposed to HIV-1 strain BaL (a strain which uses CCR5 as a co-receptor) for 4 h. Using the same 30-plex assay, cytokine, chemokine, and growth factor output from the epithelium

Figure A1.5A

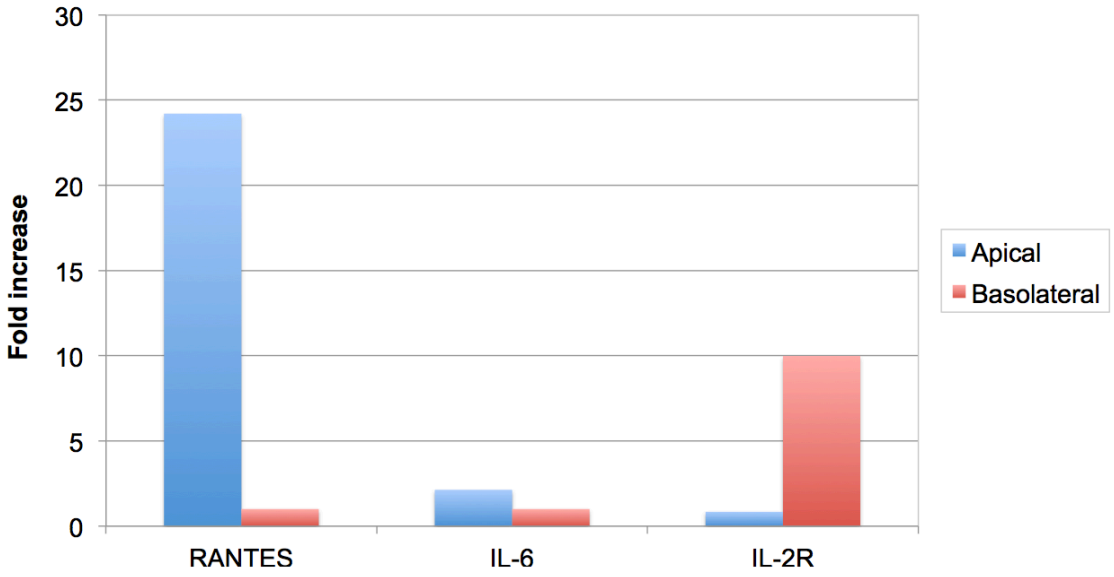
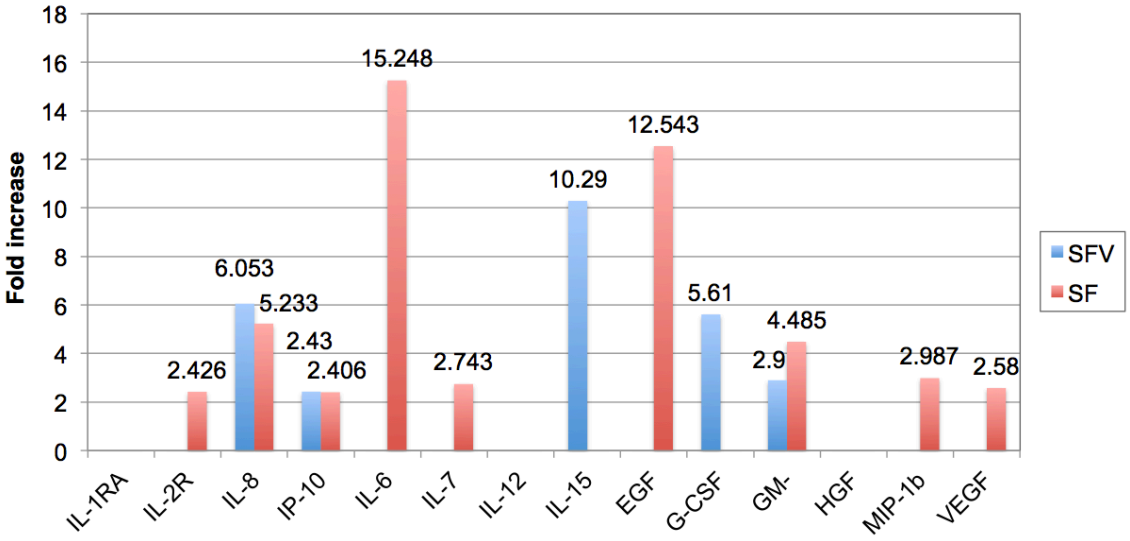

30
25
20
15
10
5
0
Fold increase
RANTES
IL-6
IL-2R
Apical
Basolateral

Figure A1.5B


Fold increase
18
16
14
12
10
8
6
4
2
0
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12.543
10.29
6.053
5.233
5.61
4.485
2.43
2.426
2.406
2.9
2.743
2.987
2.58
IL-1RA
IL-2R
IL-8
IP-10
IL-6
IL-7
IL-12
IL-15
EGF
G-CSF
GM-
HGF
MIP-1b
VEGF
SFV
SF

Figure A1.5C

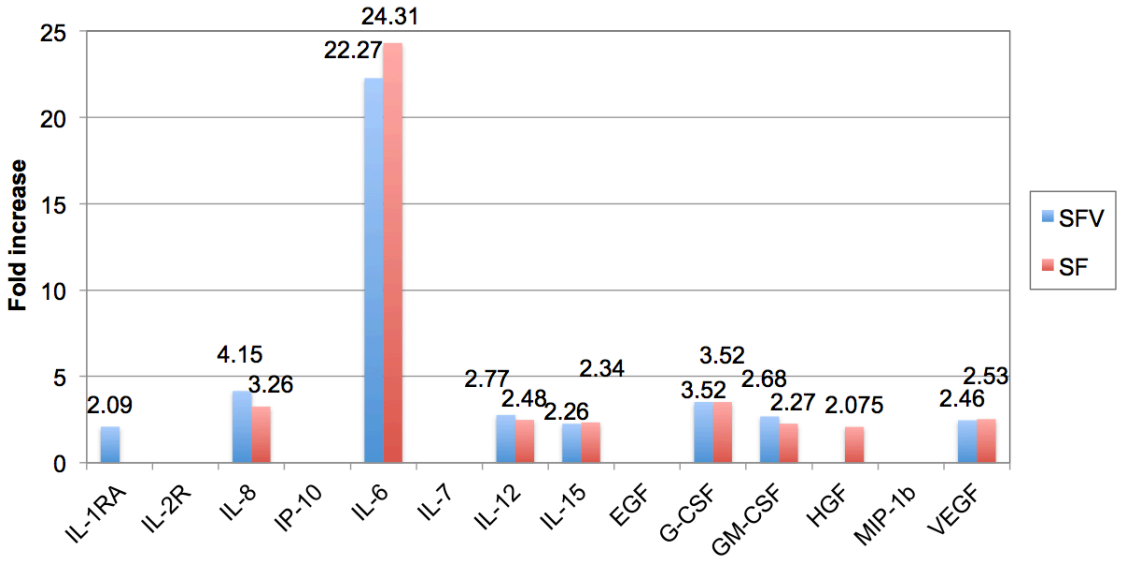

Fig. A1.5. Release of factors as a response to apical exposure to HIV-1 BaL. (**A**) Ect1 conditioned media was collected at 4 h from the apical and basolateral chambers after exposure to HIV-1 in the absence of SF. Concentration changes are expressed as fold-change relative to mock-exposed wells. (**B,C**) Ect1 conditioned media was collected at 24 h post-exposure from the (**B**) apical and (**C**) basolateral chambers after apical 4 h exposure to 25% SF in the absence or presence of HIV-1 BaL. Concentration changes are expressed as fold-change relative to mock-exposed wells. SF, semen only; SFV, uninfected pooled semen combined with HIV-1 BaL.

was measured after 4 and 24 h of incubation. The addition of apical virus to Ect1 cells most prominently induced high concentrations of RANTES in the apical chamber, with a 24-fold increase over mock-exposed monolayers at 4 h and a small increase in IL-6 (Fig. A1.5A). In contrast, the basolateral chamber, which did not contain any detectable RANTES, included a 10-fold increase in concentration of IL-2R over mock-exposed monolayers (Fig. A1.5A). By 24 h post-exposure, however, most of the factors (including RANTES) were found at concentrations less than 2-fold relative to mock-exposed levels (data not shown). These lower levels of soluble factors may be consistent with virus removal from the apical chamber at 4 h post-exposure. In summary, the release of these factors was both time- and direction-dependent.

HIV-1 BaL was also added to uninfected semen in order to determine the combined effects of SF and virus. Factors released by Ect1 cells exposed apically to SF with virus (SFV) (Fig. A1.5B and C, blue bars) were similar to those released by Ect1 cells exposed to SF alone (Fig. A1.5B and C, red bars), and most analytes were increased to the same degree relative to mock-exposed cells across both profiles. However, there was a slight upregulation of IL-1RA (~2-fold) into the basolateral chamber in the presence of virus that had not been detected in the conditioned media from cells exposed to semen alone (Fig. A1.5C). Interestingly, there were fewer factors expressed in the SFV wells compared to the semen wells in the absence of virus (Fig. A1.5B and C, blue bars). For example, HGF was increased 2-fold in conditioned basolateral media from SFV-exposed cells, but was absent from the SF-only wells (Fig. A1.5C). Similarly, IL-2R, IL-7, and MIP-1β were

present in the apical supernatants from cells exposed to SF only, but were undetectable in conditioned media subsequent to the addition of virus (Fig. A1.5B). These results indicate that virus was capable of subtly modulating the cytokine output of the epithelium, and could potentially alter the local immune response as a consequence. Further study will be required to determine whether this profile is dependent on the viral strain or origin of the epithelial cells, and is consistent with effects of the virus *in vivo*.

A1.5 Discussion

The transwell tissue culture system is an effective means to model polarized tissues. Previous investigations have relied on the transwell system for the purpose of studying other tissues and organs, such as the kidney [181] and gut [182]. In our studies, this model system has been used to recapitulate the FRT *in vitro*, with the apical chamber representing the vaginal lumen and the basolateral chamber representing the subepithelial space. This experimental design has a distinct advantage over traditional submerged culture in that it maintains the topology of the FRT in an *in vitro* setting and offers a polarized environment in which (i) the epithelial monolayers can be exposed apically to semen, mimicking semen deposition in the FRT, and (ii) the cells can be provided with nutrients from the basolateral interface during apical exposure, thereby eliminating the toxic effects that have typically been associated with SF exposure in submerged culture studies. Since semen is not regarded as cytotoxic *in vivo*, the transwell model is a better representation of the FRT relative to submerged cell culture models.

This model also provides the opportunity to examine the polarized release of immunomodulatory factors in response to semen exposure. Previous studies of the effects of semen on the epithelium lining the FRT have relied on submerged cell culture as the preferred model system. This approach is expeditious and inexpensive, since the cells grow to confluence in a matter of 3 or 4 days, compared to a 20-day growth period required for cells cultured in a transwell insert. As demonstrated in our experiments, however, cell confluence after three days does not equate to the formation of tight intercellular connections, despite the presence of a detectable ZO-1 network (data not shown). The extra culture time is necessary for the assembly of tight junction-based connections between cells and the formation of a high resistance epithelial barrier.

Experiments using the transwell culture model also revealed that the cervicovaginal monolayers are capable of releasing factors in a polarized manner in response to semen. This pattern of release varied with time, cell line identity, and direction of release. The profile of cytokine release at 4 and 24 h post-exposure across the three cell lines was predominantly pro-inflammatory, with generally low or undetectable levels of anti-inflammatory factors. Several factors were predominant early, reaching maximal concentrations at 4 h and then tapering off to undetectable levels by 24 h. Other factors that were not secreted early were detectable by 24 h, suggesting the activation of a slower pathway of *de novo* protein production and their participation in later events in the process of fertilization. Specifically, RANTES was detected only in 24 h supernatants collected from the basolateral chamber of VK2 cells. RANTES is an important

chemotactic cytokine that recruits leukocytes, including T-lymphocytes, to the site of inflammation [119]. T cells play an important reproductive role at the site of implantation by secreting LIF and M-CSF that are important in maintaining pregnancy [183]. RANTES may also be involved in the process of inducing tolerance in the FRT by controlling inflammation, possibly through the recruitment of uterine natural killer cells (uNK) that play an essential role in angiogenesis to support fetal development [119, 184, 185]. Elevated RANTES at 24 h also suggested that, despite SF removal, the epithelium remained activated and capable of releasing soluble factors, including those that had not been present during semen incubation. The sustained effect correlates with post-coitus events *in vivo*, indicating that, despite the elimination of seminal fluid from the reproductive tract within ~4 h, the epithelium maintains a sustained response by releasing factors that continue to drive local effects in the FRT.

We also observed differential release of cytokines between the apical (lumen) and basolateral (subepithelial) chambers. Of course, previous studies involving submerged epithelial cell cultures were not able to examine polarized factor release. This is meaningful, as our studies demonstrated that several analytes were limited in the direction of their release. For example, IL-4 was released strictly into the apical chamber at both early and late time post-exposure. Other factors that were expressed solely in the apical chamber included IL-13, MIG, MIP-1β, EGF, GM-CSF, and HGF; several of these factors have documented roles in promoting fertility [167, 186-188]. Several factors were released at higher concentrations in a basolateral direction compared to the apical chamber, including

G-CSF, which increased 3.5-fold in the basolateral chamber and only 1.3-fold in the apical chamber of Ect1 cells at 24 h. These results also indicate that factors may be released on both sides of the epithelium but at very different concentrations. A prominent example of this disparity is IL-8, which was increased at 24 h from Ect1 cells at 5.2-fold (6971 pg/ml) in the apical chamber but only 3.3-fold (1376 pg/ml) in the basolateral chamber. The differing concentrations of factors detected in the apical and basolateral chamber are likely correlated with the different environments of the lumen and subepithelial space in the FRT. Quantification of analytes present in the lumen using cervicovaginal lavage (CVL) samples from young women demonstrated that the FRT releases similar concentrations of factors *in vivo* as the cell lines in our model. Specifically, CVLs from women with an immature cervical epithelium contained low concentrations of RANTES (2.55 pg/ml) that were comparable to concentrations released from Ect1 (2.1 pg/ml) and End1 (1.5 pg/ml) mock-exposed apical supernatants at 24 h [189]. In addition, there were similar expression profiles for IL-6, IL-8, and MIP-1α, [189, 190]. However, there are several variables associated with the CVL findings that must be considered, including donor variability, age, sexual maturity, and the fact that lavage samples include secretions from both the vagina and cervix. This latter fact confounds attempts to correlate our observation of region-dependent factor release with the CVL study findings [189, 190]. Based on these studies, however, it is evident that the lumen expresses an abundance of chemokines at basal levels, which likely maintain a constant influx of neutrophils into the FRT in order to remove waste products produced by bacterial flora and pathogens that are

regularly encountered in the non-sterile vaginal tract [87, 114]. Interestingly, SF stimulation resulted in the production of pro-inflammatory cytokines (IFN-γ, IL-6, and IL-12) in the apical chamber at 24 h, suggesting that the lumen mounts an immune response aimed at clearing foreign antigens (in this case, semen) through the recruitment of T cells and macrophages [28]. In contrast, the basolateral (subepithelial) space was initially pro-inflammatory, but more neutral by 24 h. This effect was particularly apparent in the VK2 monolayers, which released IL-12 (6-fold), IL-6 (5-fold), IFN-γ (3-fold), IL-1β (3-fold), IFN-α (2-fold) at 4 h, but less IL-12 (2-fold), the same amount of IL-6 and IFN-α, and undetectable IFN-γ and IL-1β by 24 h. Additionally, IL-17, which was not expressed at 4 h, was increased 3.5-fold at the later time point. The shift in the release profile reflects the biphasic shift that occurs *in vivo* during the transition from inflammation to tolerance after semen deposition [87]. Together, the inflammatory factors expressed in both chambers, combined with the low levels of anti-inflammatory cytokines expressed apically and the continued expression of chemokines (such as RANTES, IL-8, MCP-1, and IP10) are responsible for the recruitment and activation of immune cell populations trafficking through the subepithelial space and mediating the shift towards a tolerogenic environment that is important for sperm survival and embryo implantation during reproduction.

Previous studies using transwell inserts have revealed some variations in the concentrations and patterns of factors released from epithelial cells. One particular study utilizing GECs showed that these cells released factors exclusively into the apical chamber [8]. In contrast, our experiments demonstrated that both

chambers were populated with factors secreted by the epithelium in the absence and presence of seminal fluid. A similar study using VK2, Ect1, and End1 cell lines in submerged cell culture determined that the Ect1 and End1 cervical cells were the most responsive at both resting state and upon exposure semen, while VK2 cells were the least active at both basal level and post-exposure [84]. It was reasoned that the non-sterile nature of the vagina causes the vaginal epithelium to become less responsive and more tolerant to semen due to the constant exposure to foreign antigens. In contrast, the cervical cells were more responsive at the level of secretion due to the lack of stimulation received at an essentially sterile location within the FRT. In our system, however, we observed an inverse of this phenotype: the VK2 cells were the most responsive at both baseline and after stimulation with seminal fluid, while the End1 cells were the least responsive, expressing the fewest number of factors in both the absence or presence of seminal fluid. This has lead us to conclude that, because the vaginal environment is continually encountering antigens, it is more responsive in order to maintain a homeostatic balance between controlled inflammation and tolerance, as compared to the upper cervix, which is less frequently exposed to pathogens and therefore produces a less robust reaction to seminal fluid. One explanation for the differences between these studies is that cells in submerged culture may be subject to feedback loops that are activated by factors released uni-directionally. These loops, which may not be effective in polarized epithelial cultures, then serve to modify subsequent output. Without proper polarization, there may also be changes at the level of factor production within the cells due to the uni-directional nature of the cells' interface

with the external environment, resulting in changes in analyte release. Differences between cells also likely contribute to the differences in results. In the GEC study, cells appeared to be much more active at a basal level, releasing 2000 pg/ml of IL-6 into the mock-exposed wells and over 3000 pg/ml after a 4 h exposure to 10% seminal plasma [8].

However, despite the differences in these studies, some consistent trends are apparent. In both our study and the GEC study, IL-6 release from the epithelium increased after semen exposure and TNF-α decreased at 24 h post-exposure. In a comparison to the submerged culture experiments, increases in IL-6, IL-8, GM-CSF, and MCP-1 were observed after semen exposure in both studies. Nonetheless, some differences in expression can also be expected due to experimental variables between studies. The submerged study exposed the cells to 10% seminal plasma (the soluble fraction of semen) for 12 h, while our experiments utilized 25% whole seminal fluid in a 4 h exposure in order to mimic post-coitus events *in vivo*. The longer exposure to semen in the other studies might contribute to differences in the measured concentrations of factors by inducing more robust and sustained response as a consequence.

A previous study of the effects of HIV-1 on TEER demonstrated that the virus – regardless of strain, length of exposure, or concentration – decreased resistance via tight junction breakdown induced by gp120 interactions with the epithelial surface [191]. In our experiments, we sought to examine alterations in cytokine output during incubation with virus. HIV-1 BaL exposure induced a very limited secretory response, with only 2-fold or greater expression of RANTES, IL-

6, and IL-2R at 4 h post-exposure. Exposure to virus in the presence of seminal fluid (SFV) resulted in a slight modulation of the semen-induced profile of cytokine release relative to SF exposure alone. Although the secretion profiles were generally similar between 24 h SF- and SFV-exposed cells, factors that were released at higher levels from SF-exposed wells (IL-2R, IL-6, IL-7, EGF, MIP-1β, and VEGF) were released apically from SFV-exposed wells at concentrations less than 2-fold above controls. In contrast, releases of IL-15 and G-CSF were higher in the SFV-exposed apical media, but less than 2-fold in the SF wells. There was additional evidence of immunomodulation by the virus in the basolateral compartment, with decreases in HGF but upregulation of IL-1RA relative to cells exposed only to semen. These results suggest that epithelial exposure to HIV-1 results in a delayed reduction in inflammatory and growth factors, but a concurrent upregulated secretion of anti-viral factors (IL-15) into the apical compartment. At the same time, there appeared to be little change in factor release within the subepithelial space, which was dominated by inflammatory factors (IL-6 and IL-12) and chemokines. The caveat to these results is that these effects were produced by combining cell-free HIV-1 with seminal fluid from healthy donors, which is likely not equivalent to semen from HIV-1-infected donors. Indeed, comparisons of semen from uninfected and HIV-1-infected donors have revealed significant differences in immunomodulatory factor content [6], suggesting differences in the effects of HIV-1-infected semen on the FRT epithelium.

Our analyses indicated that semen is generally pro-inflammatory, which is in agreement with other reports [6-8]. However, published evidence indicates that

the factor content is variable across individuals and may be influenced by disease state, including infection by STD pathogens and infertility. It is therefore likely that the epithelial response will be shaped by the contents of semen. When compared to other studies, several factors were detected at similar levels in the pooled seminal fluid of our healthy cohort. IL-2, IL-7, IL-10, VEGF, EGF, G-CSF, and TNF-α were present at similar concentrations ranges. The concentration of GM-CSF was similar to most reported values except for the GEC study. In comparison, IL-6, IFN-γ, IFN-α, and MCP-1 were on the higher end of the reported range of values in our study, while RANTES was present in pooled semen at the low end of the published concentration range. While factors were fairly consistent among the healthy males, recent studies using HIV-1-infected semen indicated that content differed between uninfected males and also varied by the stage of infection. Additionally, semen content can vary relative to the concentrations in the peripheral blood [6]. These changes could be important in the context of HIV-1 transmission within the FRT, and even with respect other sexually transmitted pathogens. The cytokines released by the epithelium in response to factors in semen during intercourse could shape the local immune response, thereby enhancing or hindering viral transmission. Immune cell recruitment, activation, and differentiation in response to epithelial stimulation by semen may be especially important as the type of cell and state of activation or differentiation could result in the influx of target cell populations with greater susceptibility to HIV-1 infection.

The transwell culture system is an inexpensive alternative to more complex modeling methods. Current methods, such as the use of MatTek cell cultures

(MatTek Corp., Ashland, MA) or the culture of *ex vivo* cervical tissue chips procured during biopsies, represent important advances in modeling the FRT. These tissues allow for the full complexity of the reproductive tract to be maintained, including important cell populations not represented in submerged or transwell cultures, such as Langerhans cells or secretory epithelial cells. Although these systems are valuable for the experimental designs they can accomodate, they also have their own limitations. Because of the cost of the MatTek cultures, the pre-made culture systems are more suited to smaller, more focused validation of experimental results obtained in less specialized model systems. Similarly, experiments using biopsy-derived tissue chips are limited by tissue availability, donor variability, and the potential for contamination during extended maintenance. Additionally, the complexity of these models can make it difficult to determine minute changes in the epithelium or the contribution of each cell type to the observed effects. For studies such as those described herein, the transwell culture model provides a transition between simple, submerged cell culture and more complex *in vitro* and *ex vivo* tissue-based systems.

These results provide evidence to support two major claims. First, semen is not an inert vehicle and is capable of directly affecting the FRT epithelium in a time-, direction-, and tissue-dependent fashion. Interestingly, our experiments using the transwell model system have also demonstrated a secondary effect of semen in the FRT: increased epithelial barrier function (Appendix Chapter 2). Second, the FRT epithelium likely plays an important role in shaping the ensuing immune response to SF through cytokine secretion. The epithelial response has been

shown to be important in the context of reproduction, shaping a biphasic shift from inflammation to tolerance. The changes in the local immunological environment will likely impact events that take place in the FRT during HIV-1 transmission. In future studies, it will be necessary to evaluate the effects of these local cytokines and chemokines on subepithelial immune cell populations in order to investigate their effects on the overall risk of HIV-1 transmission.

Appendix Chapter 2

Factors in human seminal fluid reduce epithelial permeability by increasing integrity of epithelial tight junctions

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A2.1 Abstract

Factors in seminal fluid (SF) that have integral roles in reproductive biology may also alter the ability of HIV-1 to penetrate the cervicovaginal epithelium and infect target immune cells. We hypothesized that one consequence of SF exposure may be alterations in cervicovaginal epithelial tissue tight junction integrity, resulting in changes in epithelial barrier function with respect to HIV-1. To address this possibility, human cell lines derived from three different regions of the female reproductive tract – vagina, ectocervix, and endocervix - were cultured in a transwell cell culture system to form polarized epithelial monolayers. Changes in barrier integrity were measured over time by monitoring transepithelial electrical resistance (TEER). Apical exposure to SF caused a rapid and sustained increase in epithelial integrity across all cell lines, as well as a decrease in permeability. Further investigation revealed that a combination of calcium, lipids, and soluble proteins in seminal fluid were responsible for inducing these changes through a specific apical interaction with reproductive-derived cell lines. In the context of HIV-1, the virus did not alter resistance, but preliminary data indicates that seminal fluid may decrease viral permeability as a consequence of the increases in tight junction expression. These results suggest that factors present in SF may initially protect the female reproductive tract from HIV-1 infection during sexual intercourse. Ongoing studies are investigating the mechanism(s) by which SF affects epithelial cell tight junction integrity as well as the changes in resistance induced in the presence of seminal fluid from HIV-1-infected men.

A2.2 Introduction

Seminal fluid (SF) is often assumed to function as a simple, passive transport vehicle for the delivery of sperm into the female reproductive tract (FRT) during fertilization. However, studies have revealed that semen may be far more complex than initially assumed [42, 84]. The majority of what is understood about semen has been garnered from studies of human infertility and animal husbandry, which have established that the process of embryogenesis is largely governed by the molecules and cells contained in semen, including cytokines, hormones, enzymes, and growth factors. During reproduction, these are intended to modulate the FRT immune response to seminal fluid after it has been deposited in the vaginal tract, priming the uterus for successful fertilization and subsequent embryo implantation [87]. Changes in concentrations of immunomodulatory factors have been associated with adverse events, resulting in the clearance of seminal constituents by the female immune system and rejection of the embryo [87, 114]. This function of semen, however, is not just important to fertility, but also potentially operative during transmission of sexually transmitted disease (STD) pathogens, such as the human immunodeficiency virus type 1 (HIV-1).

Roughly half of the global HIV-1 infected population is comprised of women, who have been primarily exposed through male-to-female heterosexual transmission [2, 174]. Despite over 30 years of study, the mechanisms that underlie the sexual transmission of HIV-1 are incompletely understood. A better understanding of the events that occur during transmission is fundamental to

developing strategies that effectively reduce or eliminate the risk of acquiring HIV-1.

Like the early infertility studies, studies of HIV-1 transmission have traditionally overlooked the participation of semen, reducing it to a static medium that deposits virus into the vaginal environment. Many studies of HIV-1 transmission – investigations of microbicide toxicity, female mucosal immunity, and local virus dissemination – have focused primarily on the female without consideration for the role of semen. Understanding the contribution of semen in the transmission of HIV-1 is gaining importance as researchers become aware of the potential active role it may play. These studies are critical in light of the recent demonstration that virus can persist in seminal fluid despite effective highly active antiretroviral therapy (HAART) [192].

Knowledge of the physiology of the FRT is also very important in understanding its interaction with seminal fluid. During heterosexual intercourse, semen and pathogens first encounter the epithelial lining of the FRT, which forms a mechanical barrier [148]. The epithelium has an innate protective quality conferred by the presence of apical tight junction protein complexes that connect adjacent cells and create a watertight seal that regulates the passage of substances between the cells [170, 193]. The number of tight junctions, which is regulated, in part, by the levels of tight junction protein expression, establishes the strength of the epithelial layer. Within the FRT, the expression of tight junction proteins varies anatomically, from very low expression in the vagina to increased expression in the upper reproductive tract [83]. Tight junctions serve as a barrier,

providing protection against STD pathogens such as HIV-1. If this barrier is disrupted, HIV-1 may be able to penetrate the subepithelium where it can access and infect susceptible CD4+ T-cell populations [98, 148].

SF is complex in composition and function, with studies indicating that semen has both inhibitory and enhancing effects on HIV-1 infection. More specifically, semen deposition in the female reproductive tract induces a pH change from acidic to neutral, a natural process that may prevent destruction of the virus and allow it to persist in the normally harsh, low pH vaginal environment. Conversely, semen has also been shown to harbor intrinsic anti-viral activity mediated primarily by other naturally occurring cationic peptides, suggesting that charge is important in antiviral activity [194]. Ultimately, the same conditions that regulate whether sperm are protected or cleared can also modulate transmission. If other factors found in SF are involved in either directly or indirectly modulating the integrity of the epithelial barrier, the first step in establishing an infection may potentially be driven by SF. For example, SF is rich in divalent metal cations, such as calcium and zinc [32, 161], which have been shown to play key roles in epithelial integrity at other mucosal sites, such as the gut, by increasing tight junction expression in order to regulate absorption. On the other hand, seminal fluid also contains IFN-γ, a factor that has been shown to increase gut permeability by inducing internalization of tight junction complexes [26]. Based on such studies, we were prompted to further investigate the effects of semen exposure on the epithelial lining of the FRT. Specifically, we sought to determine the effects of semen on epithelial integrity and identify factors in semen that may be mediating

these changes. The results of these studies will have clear relevance to a greater understanding of HIV-1 transmission within the FRT.

A2.3 Materials and Methods

A2.3.1 Cell Line maintenance

Experiments were performed using cell lines derived from four regions of the human female reproductive tract: uterine epithelial cells (HEC-1-A), endocervical cells (End1/E6E7), ectocervical cells (Ect1/E6E7), and vaginal keratinocytes (VK2/E6E7) (ATCC, Manassas, VA). Prior to seeding, cells were grown and maintained in T150 tissue culture flasks (Grenier Bio One, Monroe, NC) and incubated at 37°C with 5% CO_2. HEC-1-A cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) 1X (Cellgro, Manassas, VA) supplemented with 10% deactivated fetal bovine serum (FBS) (Gemini Bio-products, West Sacramento, CA), 0.05% sodium bicarbonate (Cellgro, Manassas, VA), 40 µg/ml of penicillin/streptomycin and kanamycin sulfate (Cellgro, Manassas, VA). Ect1 cells were cultured using the Keratinocyte Serum-Free Medium kit (Gibco, Grand Island, NY), which includes 50 µg/ml bovine pituitary extract (BPE) and 0.1 ng/ml epithelial growth factor (EGF) supplements, with 40 µg/ml penicillin/streptomycin solution added. End1 and VK2 cells were cultured in the Keratinocyte-SFM supplemented with 0.4 mM calcium chloride (EMD Millipore, Billerica, MA).

P4-R5 MAGI cells (AIDS Reagent Program, Germantown, MD) are a non-tight junction-expressing, HeLa-derived, cervical epithelial cell line that has been stably transformed to express β-galactosidase under the HIV-1 LTR promoter during HIV-1 infection. The cells were cultured in DMEM supplemented with 10% FBS, 7.5% (w/v) sodium bicarbonate, penicillin/streptomycin solution 50X, 5000 µg/ml kanamycin sulfate, and 1 µg/ml puromycin (Sigma-Aldrich, St. Louis, MO).

A2.3.2 Transwell culture system

Cell lines were cultured into confluent, polarized monolayers by seeding into the apical chamber of a 6.5 mm, 0.4 µm pore, polyester (PET) transwell insert in a 24-well receiver plate (Corning, Corning, NY). All cell lines were seeded at a density of 1.5×10^5 cells per well in 200 µl of media in the apical chamber and 600 µl in the basolateral chamber. Cells required 14 to 21 days of maintenance for the development of monolayers with high resistance (see below).

A2.3.3 Transepithelial electrical resistance

Epithelial monolayer confluence was monitored by measuring transepithelial electrical resistance (TEER or TER), which is an indirect measure of tight junction formation between cells. Using an EVOM2 voltohmeter and STX2 chopstick electrode (World Precision Instruments, Sarasota, FL), resistance was read once daily, in ohms, during the growth phase. Monolayers were determined to be fully confluent once the resistance readings plateaued for 2 or 3 consecutive days.

A well containing media only was also measured at each time point to provide background resistance readings. Monolayer resistance was calculated by

subtracting the background value from the experimental reading and then expressing that value as a percentage of the average value for mock-exposed wells (monolayer exposed to media only).

Due to the differential expression of tight junctions by each cell line, polarized monolayers plateaued at different resistance levels: HEC-1-A cells at ~350 Ω; End1 cells at ~250-300 Ω; Ect1 cells at ~220-250 Ω; and VK2 cells at ~220 Ω. These measurements were comparable to measurements recorded in other studies using these cell lines [148, 175].

A2.3.4 Seminal Fluid

Seminal fluid (SF) was purchased as 10 ml of semen pooled from six healthy donors between 26 and 34 years of age (Lee Biosolutions, St. Louis, MO). Specimens were shipped overnight on dry ice, immediately divided into 500 µl aliquots, and frozen at -20°C upon arrival. In all of the experiments described, SF refers to whole, crude semen containing sperm. In experiments using seminal plasma (SP), semen was centrifuged for 15 min at 3000g. The liquid portion containing soluble factor was collected and referred to as “SP”. The resulting pellet, which contained sperm and other cellular components, was resuspended in an equivalent volume of PBS and labeled as the “sperm” fraction. SP and sperm fractions were diluted to the same 25% concentration as SF.

A2.3.5 Permeability

Cells in transwell inserts were exposed apically for 4 h to 25% SF diluted in culture media, then aspirated and washed apically and basolaterally three times with media. The apical chamber was loaded with 4 mg/ml of 70 kDa dextran-FITC

(Sigma-Aldrich, St. Louis, MO) diluted in keratinocyte-SFM media (which is phenol-red free, thereby eliminating the background autofluorescence that is characteristic of phenol red-containing DMEM culture medium), while the basolateral chamber was filled with keratinocyte-SFM alone. A time course experiment (data not shown) determined that maximum dextran-FITC penetration into the basolateral chamber occurred by 30 min of incubation in the dark at 37°C. As an additional control, dextran-FITC was loaded into an empty, cell-free transwell insert in order to account for background diffusion through the porous membrane. After incubation, aliquots from the basolateral chamber were transferred in duplicate to an empty 96-well plate with black, opaque walls and a clear bottom (BD, San Jose, CA). Media only controls were also added to several wells to serve as background. Migration of fluorescent molecules from the apical to the basolateral chamber was then measured using a Fluorskan fluorometer with filters set at 485 nm to 520 nm absorbance (Thermo Fisher Scientific, Waltham, MA). Relative permeability was calculated by subtracting the average media-only well reading, which represents autofluorescent background, from each of the experimental reading. The resulting numbers were then set relative to the empty well by dividing the experimental reading by the empty well and expressing the final value as percent permeability relative to an empty well.

A2.3.6 Immunofluorescence

Transwells were prepared for immunofluorescence by washing gently with HBSS to remove any residue (Cellgro, Manassas, VA). Cells in the transwells were then fixed by incubating in a 2% paraformaldehyde (PFA) (Alfa Aesar, Ward Hill,

MA) solution diluted in HBSS added to the apical chamber only. The monolayers were incubated for 10 min at room temperature (RT) in a dark laminar flow hood, followed by a second incubation with 4% PFA apically for 20 min at RT in the dark. After fixing, the cells were washed and permeabilized in a 0.5% solution of Triton X-100 (Sigma-Aldrich, St. Louis, MO) in both the apical and basolateral chambers of the transwells. The plates were incubated at RT in the dark for 1 h and then washed at least three times with HBSS, or no traces of detergent remained. For staining, the cells in transwells were incubated in a 50 µg/ml concentration of ZO-1 antibody conjugated to Alexa Fluor 488 (Life Technologies, Grand Island, NY) diluted in a 2% donkey serum/HBSS solution (Jackson Laboratory, Bar Harbor, ME). Prior to the addition of antibody, transwell inserts were transferred from the receiver plate into a 6-well plate (BD Falcon, San Jose, CA) so that the inserts were sitting flat on the bottom of the culture well, thereby preventing antibody leakage through the membrane. During incubation, the plates were wrapped in foil and placed at 37°C for 2 h, and stored at 4°C overnight. The next day, the wells were washed and stained with a 1:100 concentration of 4',6'-diamidino-2-phenylindole (DAPI) (Life Technologies, Grand Island, NY) diluted in HBSS for 2 min. The DAPI stain was removed by washing three times with HBSS, followed by a wash with sterile diH_2O (Cellgro, Manassas, VA) to remove HBSS crystals. In order to visualize the cells, the membranes were carefully excised from the transwell inserts while they were still moist, placed onto slides (Thermo Fisher Scientific, Waltham, MA), and then sealed under coverslips (VWR, Radnor, PA). Staining was visualized using an Olympus 1X81 inverted fluorescence microscope

(Olympus America, Center Valley, PA) and images were captured with Slidebook 5.0 Digital Microscopy software (Intelligent Imaging Innovations, Denver, CO).

A2.3.7 Semen exposure and heat denaturation studies

During acute exposure, cell lines cultured into confluent monolayers in the apical chamber of transwells were incubated with either 25% SF, 25% SP, or 25% sperm fraction diluted in either media or PBS (as indicated). Changes in TEER were measured every 5 min for the first half hour and then every 30 min for the remaining 4 h exposure. In a long-term exposure, additional TEER readings were taken daily out to six days, with media changes every other day. For heat denaturation studies, the semen was heat inactivated prior to the experiment in a water bath at either 55°C for 1 h or 65°C for 20 min (both are commonly used methods) and stored at -20°C.

A2.3.8 Tight junction breakdown studies

Confluent monolayers were incubated apically or basolaterally (as indicated) with either 4 mM EDTA (Cellgro, Manassas, VA), 4mM EGTA (Boston Bioproducts, Ashland, MA), 1.4 M of mannitol (Sigma-Aldrich, St. Louis, MO), or 0.1% C31G, diluted in media as positive controls for tight junction break down. Changes in TEER were measured during the acute time course described previously. In the tight junction recovery studies, the monolayers were pre-incubated with 4 mM EDTA or EGTA for 30 min, followed by a gentle wash with media, and then either new media or 25% SF was added. Additional readings were taken daily out to 72 h post-exposure.

A2.3.9 Metal cation studies

Monolayers were exposed to physiological concentrations of metal ions naturally found in seminal fluid. Cells were incubated apically with either 1 mg/ml each of KCl (EMD Millipore, Billerica, MA), $MgCl_2$ (VWR, Radnor, PA), $ZnCl_2$ (EMD Millipore), $CaCl_2$ (EMD Millipore), or a metal ion mixture of all four, and measured for changes in TEER up to 4 h.

A2.3.10 TGF-β and pH resistance studies

HEC-1-A and Ect1 monolayers were incubated apically with TGF-β at either 70 ng/ml, representing the high end of the average physiological concentration of TGF-β present in seminal fluid from healthy males [70], or 2 ng/ml, a low concentration control, and measured for changes in TEER over the course of 4 h. In pH acid-activation studies, the cells were first assessed for changes in response to a 1 h incubation in a pH titration ranging from a pH of 8.2 to 4.0. The range of pH concentrations were achieved by adjusting the media with sodium hydroxide and hydrogen chloride (J.T. Baker, Center Valley, PA) in a drop-wise manner. Changes in TEER were measured over the 1 h time course and then cell viability was assayed as described below. Following the initial optimization experiment, HEC-1-A and Ect1 monolayers were exposed to SF in a transition model: transwells were pre-incubated for 1 h at pH 4, then 25% SF was added and TEER readings were recorded for 1 h. A 25% SF control well was also measured in which semen was added to the normal, neutral culture media (pH 7.2).

A2.3.11 Cytotoxicity assay

Changes in cell viability after semen, virus, or low pH exposure were assessed spectrophotometrically to detect the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by dehydrogenase enzymes in the mitochondria, a process that results in the production of purple formazan crystals [195]. After washing the transwells with HBSS, MTT powder (Sigma-Aldrich) dissolved in media at a concentration of 7.5 mg/ml was added to the apical chamber of each transwell and fresh media added to the basolateral chamber. The plates were incubated for 45 min to 1 h at 37°C and then aspirated. The formazan crystals trapped within the cells were solubilized in acidic isopropanol by shaking at room temperature for 20 min. The dissolved solution was then transferred to a clear, flat-bottomed 96-well plate (BD Falcon, San Jose, CA) and read on a multiskan colorimeteric plate reader (Thermo Scientific, Waltham, MA). The calculated absorbance is proportional to cellular viability and inversely proportional to any toxicity due to semen exposure [196].

A2.3.12 TLR2 interaction studies

Cell lines cultured into confluent monolayers in the apical chamber of transwells were measured for TEER then pre-incubated for 1 h in 20 µg/ml of Toll-like receptor 2 (TLR2) monoclonal antibody diluted in media (Invivogen, San Diego, CA). Following pre-incubation, SF was added to the TLR2 antibody solution in the transwell at a concentration of 50% in order to give a final chamber concentration of 25% SF in the presence of 10 µg/ml of TLR2 antibody. TEER

measurements were taken as out to 24 h. As a control, wells containing 20 μg/ml TLR2 antibody only were also measured over the time course. In order to ensure that the monolayers expressed TLR2 when cultured in this model, peptidoglycan (PGN) (Sigma-Aldrich, St. Louis, MO), a bacteria-derived, known TLR2 agonist, was included as a positive control. PGN diluted to 10 μg/ml as a suspension in media was then added to the apical chamber of the transwells either alone or in the presence of the TLR2 antibody and TEER measurements were monitored over the 24 h time course.

A2.3.13 HIV-1 permeability studies

Monolayers of HEC-1-A, Ect1, and End1 cells cultured to confluence in transwells were exposed apically for 4 h to either HIV-1 laboratory strains BaL (Zeptometrix, Buffalo, NY) or HIV-1 IIIB (Advanced Biotechnologies Inc, Columbia, MD), in the presence or absence of 25% SF and changes in TEER were monitored. After 4 h, the basolateral chamber supernatant was removed and incubated with P4-R5 MAGI cells, which express CCR5 and CXCR4 co-receptors to allow infection, seeded at 1.5 x 10^4 cell/well in a 96-well flatbottom plate for 48 h. After incubation the cells were assayed for β-galactosidase production as a relative measure of HIV-1 infection using the Galacto-Star *β*-Galactosidase Reporter Gene Assay System for Mammalian Cells per the manufacturer's protocol (Applied Biosystems, Bedford, MA).

A2.3.14 Statistical analysis

p-Values were calculated for the difference between pairs of treatment conditions using a two-tailed Student's *t*-test and a value of less than 0.05 was

set as the limit for statistical significance. p-Values are indicated on the figures and within the figure legends.

A2.4 Results

A2.4.1 SF exposure causes rapid increases in epithelial monolayer TEER

Studies of the polarized release of immunomodulatory factors from FRT epithelial cell lines exposed to semen (Appendix Chapter 1) established the transwell culture system as a model for the human FRT epithelium. In this model, epithelial cells are cultured in the apical chamber of a transwell insert to form confluent monolayers with a high electrical resistance characteristic of cells interconnected by tight junctions. The model system emulates the topography of the FRT epithelium, where the apical chamber represents the cervicovaginal lumen and the basolateral chamber represents subepithelial tissue. Previous studies demonstrated the release of immunomodulatory factors from SF-exposed epithelial cells in a direction-, time-, and cell type-specific manner (Appendix Chapter 1). In experiments designed to examine any adverse effects of SF on monolayer permeability, a small but significant decrease in permeability was noted after SF exposure. The following experiments explore that observation in more detail.

Previous studies have established that, once semen is deposited in the FRT, resident secretions dilute it to about 10-25% concentration, which then dissipates over the course of approximately 4 h [29]. Using these parameters, monolayers of HEC-1-A cells in transwell inserts were exposed apically to 25% or

10% SF for 4 h. SF exposure resulted in a time-dependent increase in resistance that persisted throughout the duration of exposure, reaching a peak of 237% at the highest SF concentration relative to mock-exposed cells transwells (Fig. A2.1A). Of particular note was the rapid speed at which the increase was initiated, which produced a significant and repeatable increase of 28% over mock untreated levels within 5 min of exposure. A dose-dependent response was also evident as the diluted 10% concentration of SF induced a similar rapid and sustained increase in TEER, but at a lower magnitude relative to the 25% SF (Fig. A2.1A). The scope of the dose-dependent effect was evaluated more closely in a SF titration spanning 50% to 0.1%, which demonstrated that increases in resistance were clearly dependent on the concentration of SF (Fig. A2.1B). For the remainder of the study, a concentration of 25% SF was utilized, as this concentration best represents the high end of the physiologically relevant concentrations occurring *in vivo* [87, 114].

A2.4.2 Changes in TEER persist during long-term incubation with SF

To determine the extent to which SF could exert effects on tight junctions, daily TEER was monitored over a long-term, five-day time course. Continuous incubation of HEC-1-A monolayers in 25% SF for 18 h resulted in a steady increase that peaked at 158% at the 24 h time point and remained elevated out to 48 h, despite the removal of SF (Fig. A2.2A). TEER returned to mock, untreated levels by 72 h and was maintained at this level over the remainder of the five-day time course. Similar to the acute exposure studies, the long-term effects were also dose-dependent, since 10% SF exposure produced a similar trend at a lower level

Figure A2.1A

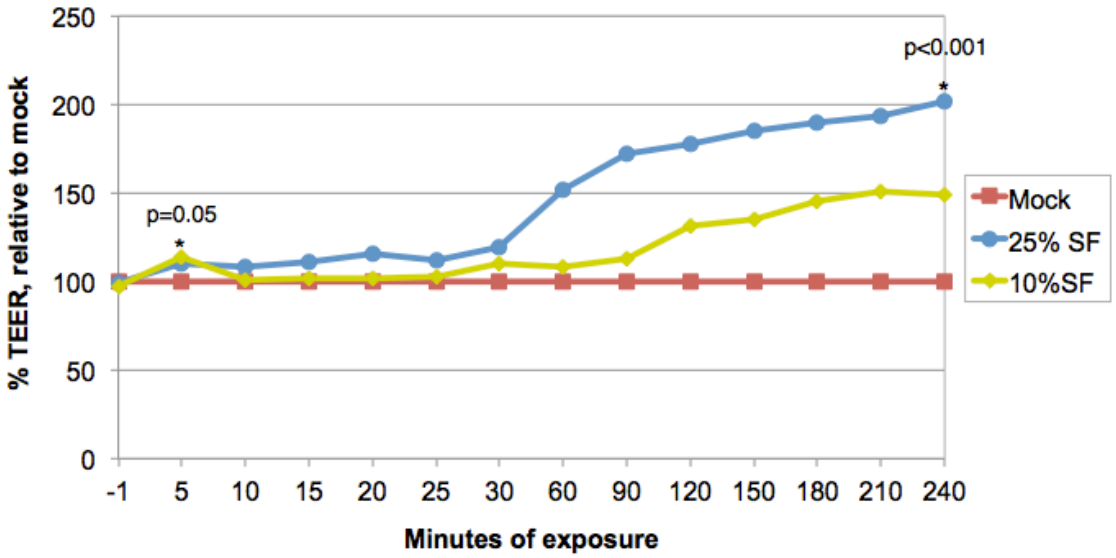
250
200
150
100
50
0
% TEER, relative to mock
p<0.001
*
p=0.05
*
-1
5
10
15
20
25
30
60
90
120
150
180
210
240
Minutes of exposure
Mock
25% SF
10%SF

Figure A2.1B

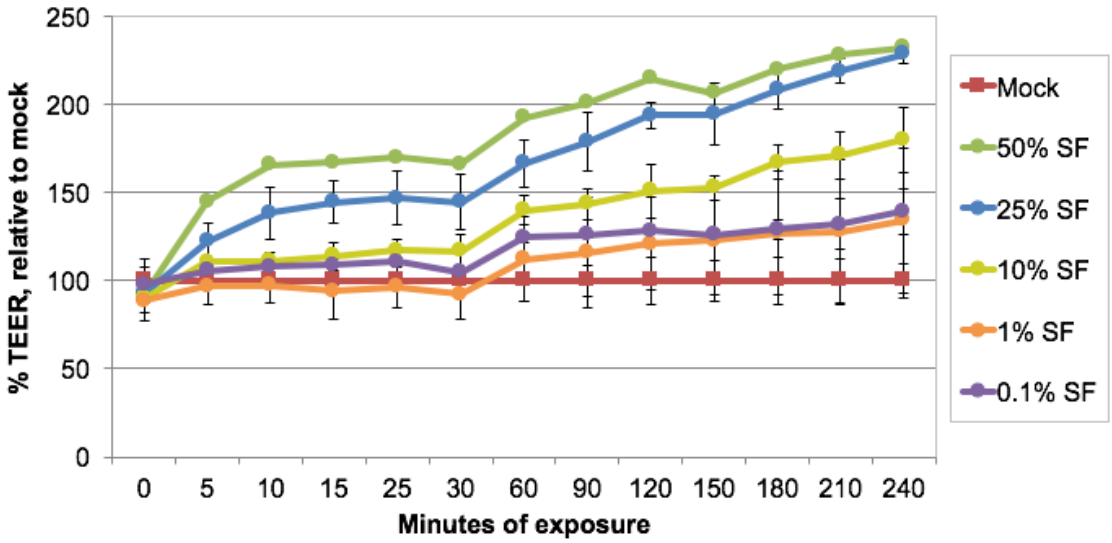

Figure A2.1 SF-induced increases in epithelial monolayer resistance are time-, concentration, and direction-dependent. (A) HEC-1-A monolayers were continuously exposed to SF for 4 h and changes in resistance were measured. (B) A titration curve of 50% to 0.1% SF dilution provided additional evidence that the degree of increase in resistance was dependent upon semen concentration.

Figure A2.2A

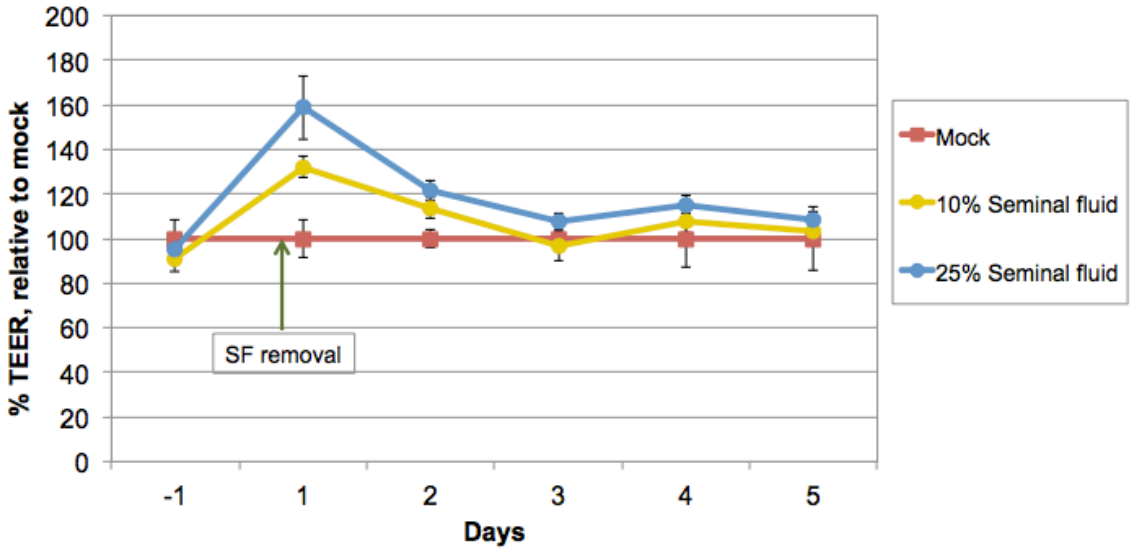
200
180
160
140
120
100
80
60
40
20
0
% TEER, relative to mock
-1
1
2
3
4
5
Days
SF removal
Mock
10% Seminal fluid
25% Seminal fluid

Figure A2.2B

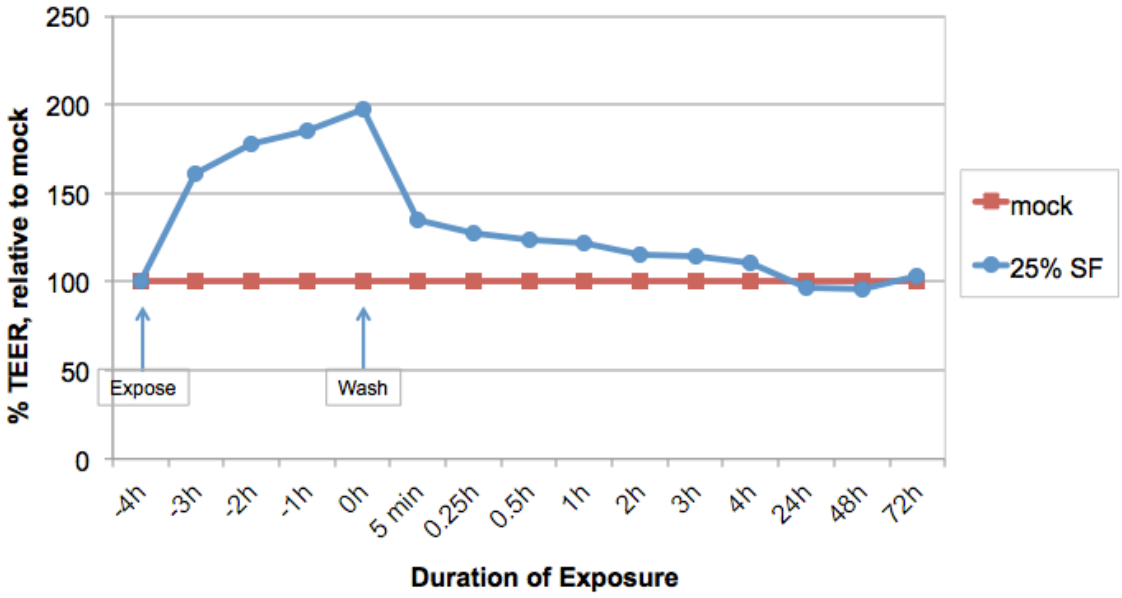

Figure A2.2 SF-induced non-permanent increases in resistance peaked at 24 h and remained elevated out to 48 h during continuous exposure. (A) Long-term incubation of HEC-1-A monolayers in either 25% or 10% SF resulted in a peak elevation at 24 h, after which point the resistance began to decrease. (B) HEC-1-A cells were pre-incubated with SF for 4 h to induce a maximal increase in resistance and then washed. After removal of SF, resistance decreased rapidly and continued a steady decline until reaching mock levels.

of resistance (Fig. A2.2B). These results suggest that, as semen is removed from the reproductive tract during the post-coital phase, the increased resistance across the epithelium is temporary and will return to mock levels within a short time period. Thus, the presence of SF is required in order to induce and maintain changes in resistance.

A2.4.3 SF-induced changes in permeability and TEER are specific to tight junction expressing cells

Changes in resistance are an indirect indication that semen is inducing changes at the level of tight junctions. To determine the specificity of the effect, P4-R5 MAGI (P4R5) cells (a HeLa-derived cell line) were cultured in transwells. Despite their epithelial and cervical lineage, these cells do not express tight junctions. After apical exposure of P4R5 cells to SF, the resistance of the cell monolayer remained at ~162 Ω, which is just above the average background level of 135 Ω (data not shown). Similarly, addition of SF to empty transwells (i.e. lacking cells) did not result in increased TEER over baseline values observed with media alone (data not shown). These observations indicated that the effects of SF were specific to tight junctions and excluded the possibility that seminal fluid was producing a false increase in resistance by physically occluding the membrane pores or filling the paracellular spaces.

The effect of SF on the epithelial barrier was confirmed by measuring changes in paracellular permeability. A fluorescently tagged tracer molecule, dextran-FITC, was utilized to measure the migration from the apical to basolateral chamber, as previously described (Appendix Chapter 1). Although the HEC-1-A

monolayers are an effective barrier at a mock level when normalized to the permeability of an empty transwell, semen exposure further reduced dextran- FITC passage to 2.7% in a repeatable and significant manner (p=0.014) (Appendix Chapter 1 and Fig. A2.3). In the non-tight junction-forming P4R5 cells, however, the low level of resistance correlated with high levels of tracer permeability, both with and without SF exposure, again demonstrating the specificity of this effect and illustrating the reciprocal relationship between permeability and TEER.

A2.4.4 Increased epithelial resistance is an exclusive activity of the seminal plasma fraction

Initial experiments used whole pooled seminal fluid, which contains both the cellular and soluble fractions produced during ejaculation. However, many studies on reproduction exclusively utilize only seminal plasma, which is the soluble fraction of semen with the cellular portion removed by centrifugation. To determine if the two main fractions comprising semen have differential effects on TEER, the seminal fluid was separated into a seminal plasma (SP) fraction containing the soluble factors, and a sperm pellet containing the cellular components. HEC-1-A monolayers were exposed apically to either 25% SF, 25% SP, or 25% sperm fraction and then the changes in TEER were measured over the 4 h time course. Interestingly, SF and SP increased TEER to the same extent in a repeatable manner, with increases beginning as early as the 5 min time point and continuing throughout the experiment (Fig. A2.4). However, the sperm component had no effect on TEER and remained at mock level. This revealed that there was no

Figure A2.3

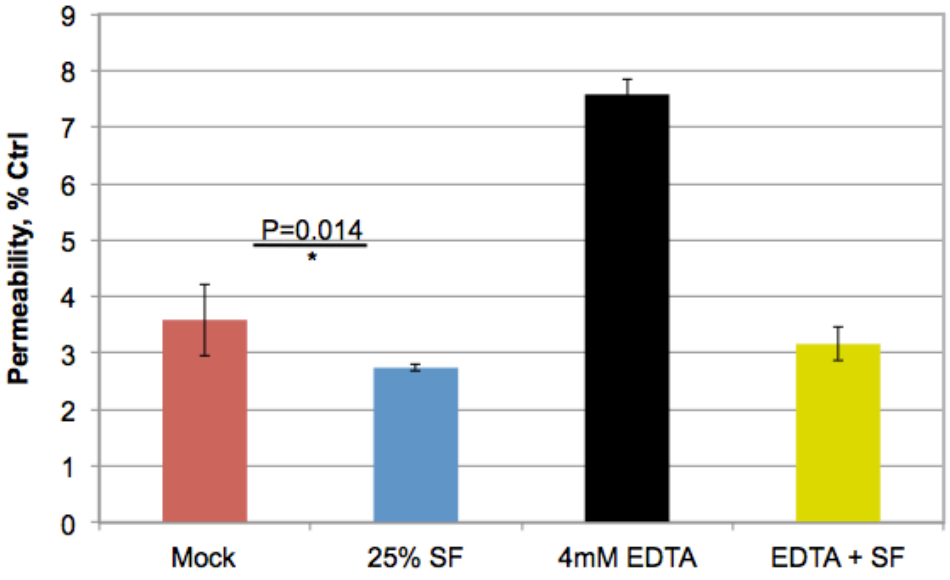

Figure A2.3 Changes in resistance during SF exposure are inversely correlated with permeability. 4 h post-exposure, HEC-1A monolayers were incubated apically with dextran-FITC to measure paracellular migration. While healthy mock monolayers were relatively impermeable due to high basal expression of tight junctions, SF exposure further significantly decreased fluorescence. EDTA exposure, which breaks down tight junctions, increased permeability but was reduced back to near mock levels during simultaneous exposure with SF.

Figure A2.4

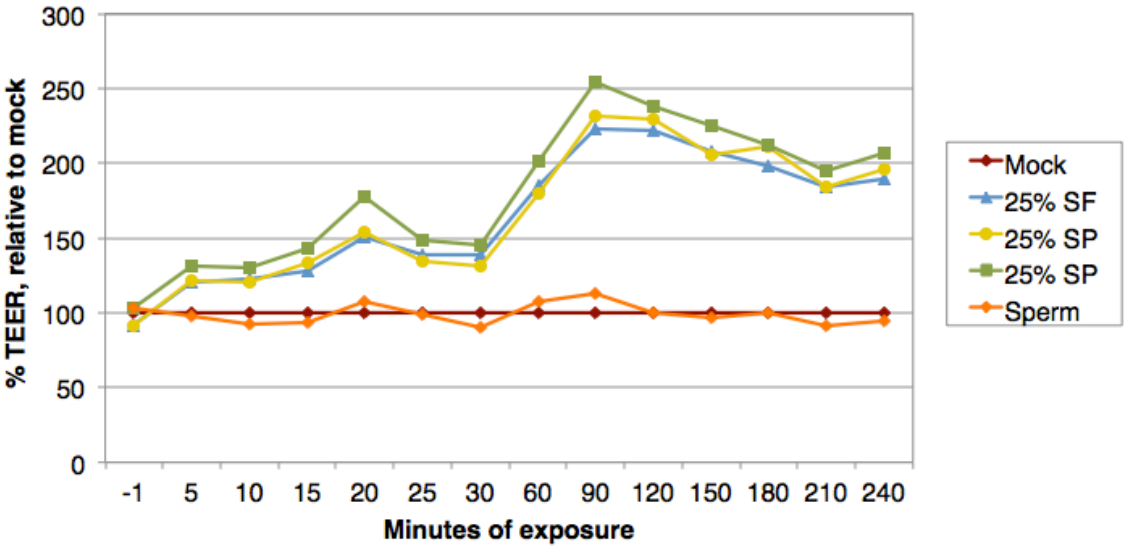

Figure A2.4 Seminal plasma contains the active component of SF. Semen was fractionated to seminal plasma (SP) and a sperm pellet containing cellular matter. Incubation with SP produced an increase in resistance that was comparable to SF, while the sperm exposed cells remained at mock level.

division of activity across the two major fractions of semen and the activity was confined solely to the SP fraction. Although the majority of previous publications utilized seminal plasma, having ruled out any deleterious effects contributed by the sperm fraction, whole SF was used throughout the remainder of this study in order to maintain a physiologically intact model.

A2.4.5 Changes in TEER are independent of the physical properties of semen

Seminal fluid is complex in both composition and property, so in order to decipher whether the effects of semen on resistance were mediated by a specific factor or due to a physical characteristic of semen as a bodily fluid, a series of experiments investigating the contribution of various physiological properties were executed.

SF is known to have mucus-like properties [32], so establish that the changes in resistance were not attributable to paracellular occlusion by the viscosity of semen, monolayers were incubated with 0.5% carrageenan, a similarly thick and viscous compound. Even with a high concentration of carrageenan, no changes in resistance were observed (Fig. A2.5A). Combined with previous data of P4R5 cells and empty wells, the contribution of semen viscosity to changes in resistance was ruled out.

In relation to viscosity, SF also has serum-like properties, so in order to determine that the effects were specific to semen and not a non-specific response of the epithelium to all serous-type fluids (such as blood serum or saliva), the same experiments were repeated with FBS substituted for SF. FBS diluted to 25% in

either HEC-1-A media (which is supplemented with 10% FBS) or in serum-free DMEM, did not induce a change in resistance (Fig. A2.5B). This also demonstrates that SF does not combine with FBS present in growth medium to enhance these effects (Fig. A2.5C). Lastly, in order to determine if any other components or supplements in the media affected resistance, SF was diluted in PBS. PBS alone did not alter resistance and use of PBS as a dilution alternative to culture media also did not modify the characteristic semen increases in resistance (Fig. A2.5D). Together, these studies on the physical characteristics of semen indicate that increased resistance is specifically attributable to soluble factors in the plasma fraction. Therefore, the next step was to identify which factor(s) in SF were mediating this effect.

A2.4.6 Different regions of the FRT differentially respond to SF

The observations from these experiments indicate that seminal fluid contains a factor that specifically alters tight junctions. Up to this point, the studies have utilized HEC-1-A cells, which are a good model as they express high levels of tight junctions, are easy to culture, and are derived from the female reproductive tract. However, HEC-1-A cells are specifically uterine epithelial cells, which although relevant to the area of study, are not the cell type primarily exposed to seminal fluid during heterosexual intercourse, nor is this a region suspected to be involved in HIV-1 transmission. This posed the question as to whether the SF-induced effects were specific to HEC-1-A cells or if it would persist using relevant reproductive cells. To address the contribution of tissue specificity in SF-mediated

Figure A2.5A

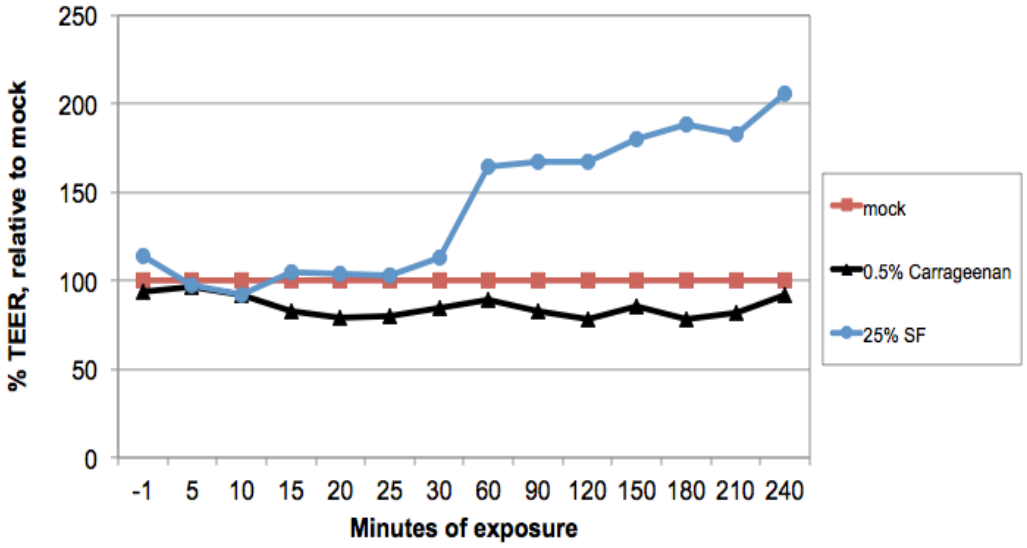
250
200
150
100
50
0
% TEER, relative to mock
-1
5
10
15
20
25
30
60
90
120
150
180
210
240
Minutes of exposure
mock
0.5% Carrageenan
25% SF

Figure A2.5B

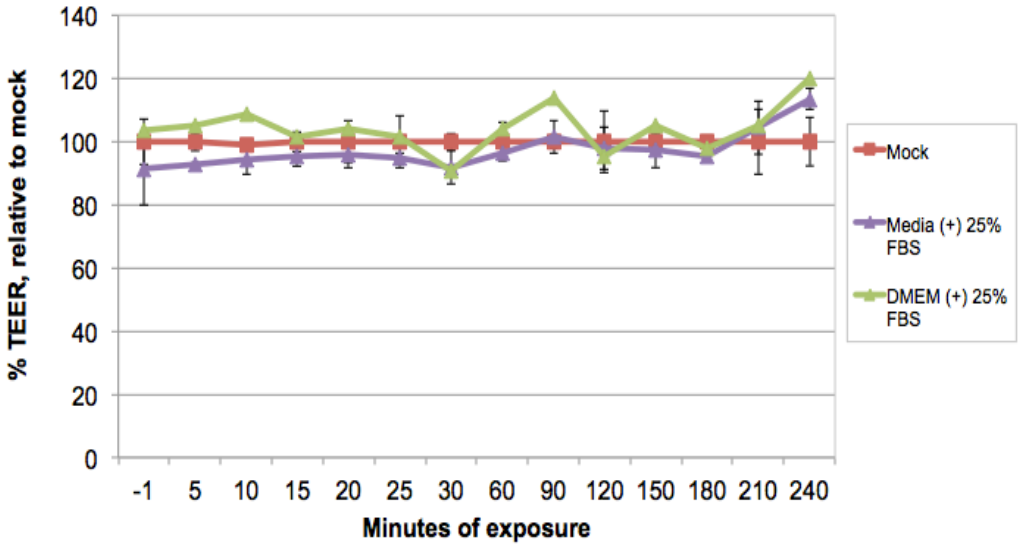
% TEER, relative to mock
140
120
100
80
60
40
20
0
-1
5
10
15
20
25
30
60
90
120
150
180
210
240
Minutes of exposure
Mock
Media (+) 25% FBS
DMEM (+) 25% FBS

Figure A2.5C

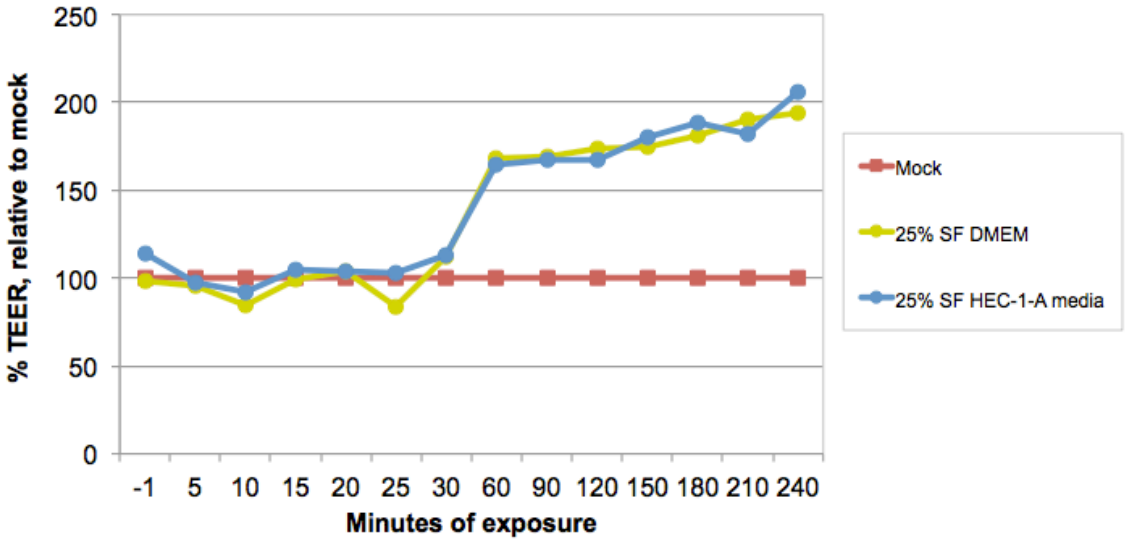
250
200
150
100
50
0
% TEER, relative to mock
-1 5 10 15 20 25 30 60 90 120 150 180 210 240
Minutes of exposure
Mock
25% SF DMEM
25% SF HEC-1-A media

Figure A2.5D

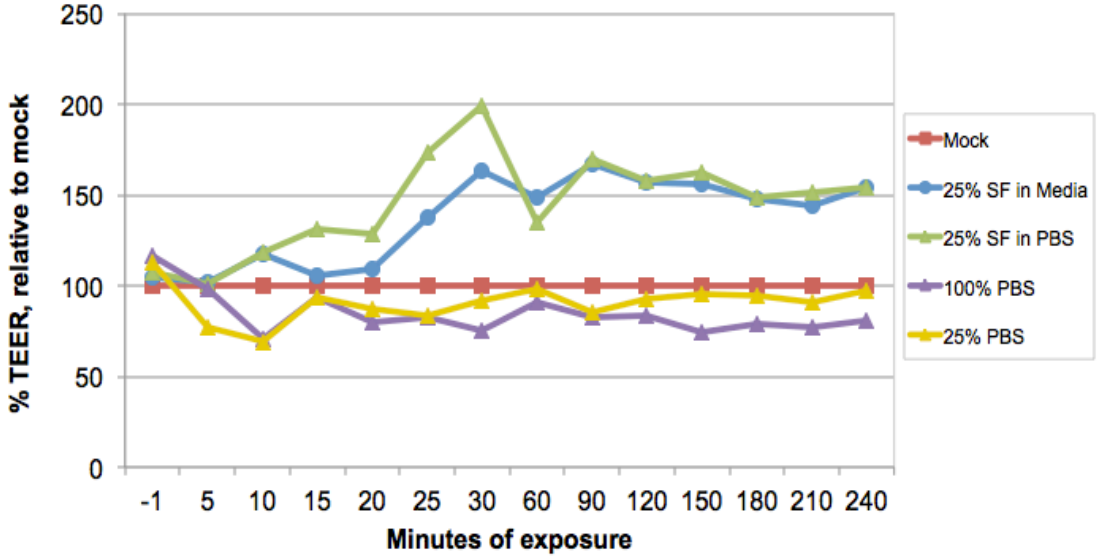

Figure A2.5 Changes in resistance are not due to physical properties of SF or outside experimental factors. A) A 25% solution of carrageenan mimicking SF viscosity excluded the possibility that SF induced an increase in resistance by physically occluding the paracellular spaces. B) A 25% FBS solution used as a substitute for semen proved that the effects on resistance were specific to SF and not a characteristic effect induced by all serum-based solutions. Similarly, any contribution to changes in resistance by C) media components were eliminated by dilution of SF in 25% serum-free DMEM or D) 25% PBS.

changes in TEER, the 4 h exposure experiments were repeated using three immortalized cell lines isolated from a single donor, representing the three main regions of the reproductive tract: VK2, vaginal keratinocytes, from the vagina; Ect1, ectocervical cells, from the lower cervix representing the transition zone; and End1, endocervical cells, from the upper cervix. All three cell lines have been characterized in literature previously, correlating tight junction expression *in vitro* with the physiology of the respective regions from which they are isolated *in vivo* [83, 175]. It has been shown that the vaginal tract has a low, disorganized pattern of tight junction expression, which supports the main function of this region as a mechanical barrier to insults, reaching upwards of 25 squamous cell layers thick in some areas [148, 191]. At the uppermost layers near the lumen, the vagina is characterized as being almost devoid of tight junction expression, which facilitates the continual sloughing of these layers and adds an additional aspect of protection [83]. When confluent VK2 monolayers cultured in transwells were exposed to 25% SF, there was a 24% increase in resistance over mock-untreated monolayers by 4 h, which, although low due to mediocre tight junction expression, was nevertheless significant and repeatable (Fig. A2.6A). In contrast, Ect1 cells, which represent the portion of the reproductive tract in which the squamous epithelium transitions into a single-layer columnar epithelial phenotype, had a greater response to SF exposure, increasing significantly 171% relative to mock-untreated (Fig. A2.6B). The greater response of Ect1 cells is due to the higher expression of tight junctions characteristic of this cell type, resulting in an organized monolayer that, despite being only one cell layer thick, is relatively impermeable. Lastly, the

End1 cells, which are also a simple columnar epithelial cell type expressing high levels of tight junctions, demonstrated a rapid increase in TEER after 5 min of SF exposure, similar to HEC-1-A cells, and continued to increase significantly out to the 4 h time point, reaching 155% relative to mock (Fig. A2.6C). Overall, each cell line increased in resistance during semen exposure in a manner consistent with the degree of tight junction expression associated with each tissue (Fig. A2.6D). These results highlight the differences in regional expression of tight junctions and the contribution of these differences to the resistance response during SF exposure.

In order to rule out any complications due to changes in viability, MTT assays of all three reproductive cell lines determined that there were no detrimental effects during SF exposure at the concentrations or time points used (data not shown).

A2.4.7 SF-induced effects are specific to reproductive cell lines

In order to determine if the effects of SF were specific to the four FRT cell lines utilized thus far or universal to all tight junction-expressing tissues, two non-FRT cell lines were evaluated. Caco-2 cells, colon derived epithelial cells, express high levels of tight junctions that peak in confluence around 800-1000 Ω. When exposed apically to SF in for 4 h, the cells increased by only 3% relative to pre-exposure readings (data not shown). The experiment was also repeated using the brain microvascular endothelial cell line hCMEC/D3 (BMECs) in place of epithelial cells, which are used to model the blood-brain barrier but express relatively low

Figure A2.6A

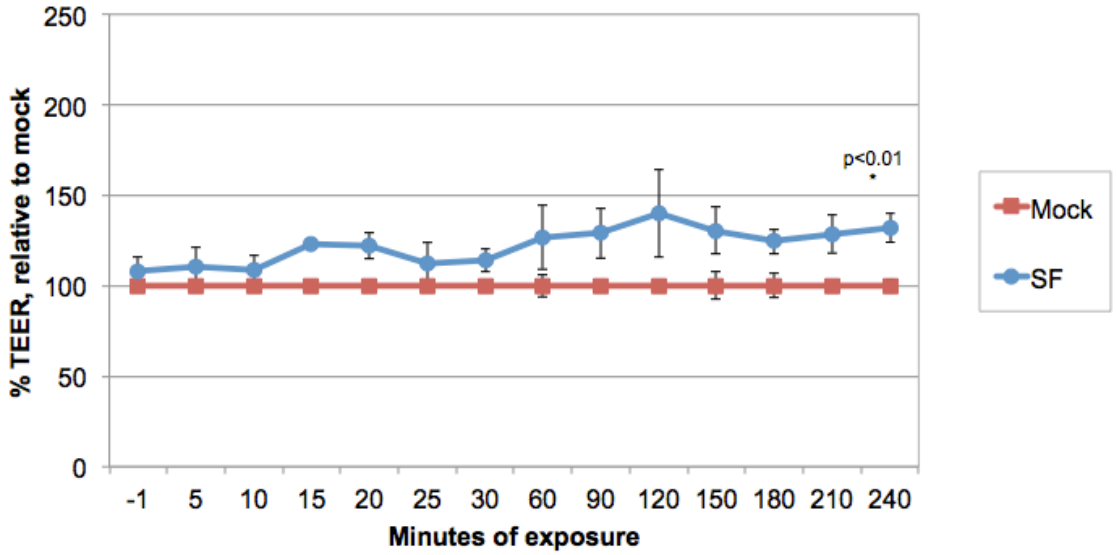
250
200
150
100
50
0
% TEER, relative to mock
p<0.01
*
-1
5
10
15
20
25
30
60
90
120
150
180
210
240
Minutes of exposure
Mock
SF

Figure A2.6B

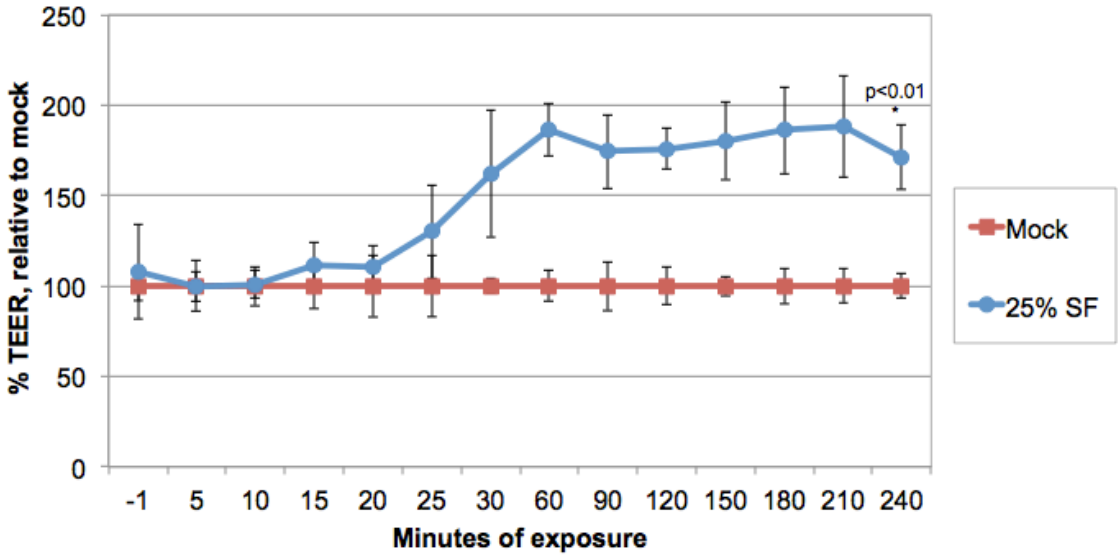
250
200
150
100
50
0
% TEER, relative to mock
p<0.01
*
-1
5
10
15
20
25
30
60
90
120
150
180
210
240
Minutes of exposure
Mock
25% SF

Figure A2.6C

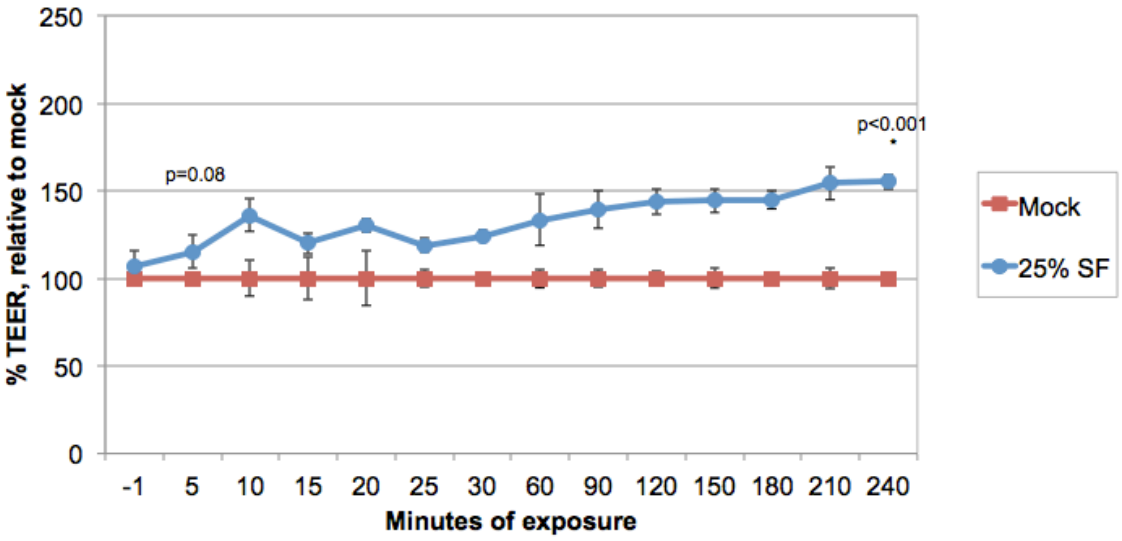
250
200
150
100
50
0
% TEER, relative to mock
p=0.08
p<0.001
*
-1
5
10
15
20
25
30
60
90
120
150
180
210
240
Minutes of exposure
Mock
25% SF

Figure A2.6D

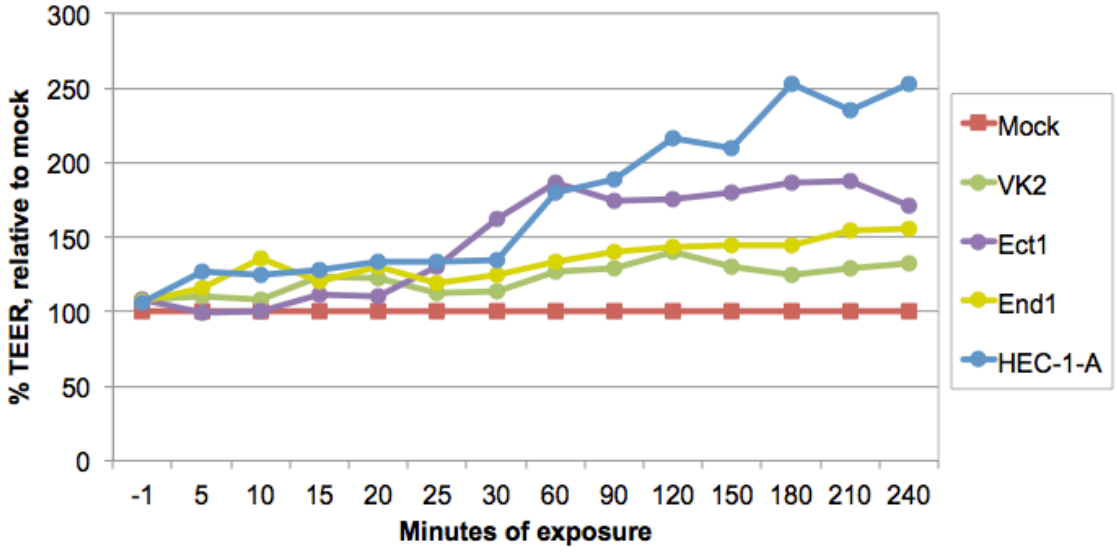

Figure A2.6 Cells derived from different regions of the FRT increase in resistance as a response to SF at varying degrees, dependent upon the characteristic tight junction expression associated with the tissues *in vivo*. (A) VK2, (B) Ect1, and (C) End1 monolayers incubated with SF for 4 h were measured for changes in resistance. Each cell line demonstrated significant increases in resistance by 4 h of SF exposure; End1 responded the most rapidly, with a significant increase by 5 min of exposure. D) Although each cell line was derived from a different region of the FRT, each responded to SF in a time-dependent manner.

levels of tight junctions. Like the Caco-2 cells, the BMECs did not respond to SF, which was determined by both TEER and permeability to dextran-FITC (data not shown). Unexpectedly, these studies indicate that the effects of SF on resistance are not universal to all tight junction-expressing cells and that the semen factor responsible may be acting in a specific manner with FRT-derived epithelial cells.

A2.4.8 EDTA abrogates SF-mediated changes in TEER

To better understand the mechanism of the effects of SF on resistance, EDTA was introduced into the model as a positive control, as it is widely utilized and well characterized for its ability to break down tight junctions [197]. In the 4 h exposure transwell assay, EDTA produced a predictable, time-dependent and dose-dependent decrease in resistance (Fig. A2.7A). Interestingly, when the monolayers were exposed simultaneously to a mixture of 25% SF and 4mM EDTA, the effects of SF were abrogated (Fig. A2.7A) in a dose-dependent manner with decreasing concentrations of EDTA (Fig. A2.7B). The changes in resistance were also consistent with changes in permeability. As EDTA broke down tight junctions and reduced TEER, there was a significant 4% increase in dextran-FITC detection in the basolateral chamber as compared to mock monolayers (Fig. A2.3). In contrast, in the presence of SF, the effects of EDTA on permeability were restored back to nearly mock levels (3.1% and 3.5%, respectively) (Fig. A2.3).

Figure A2.7A

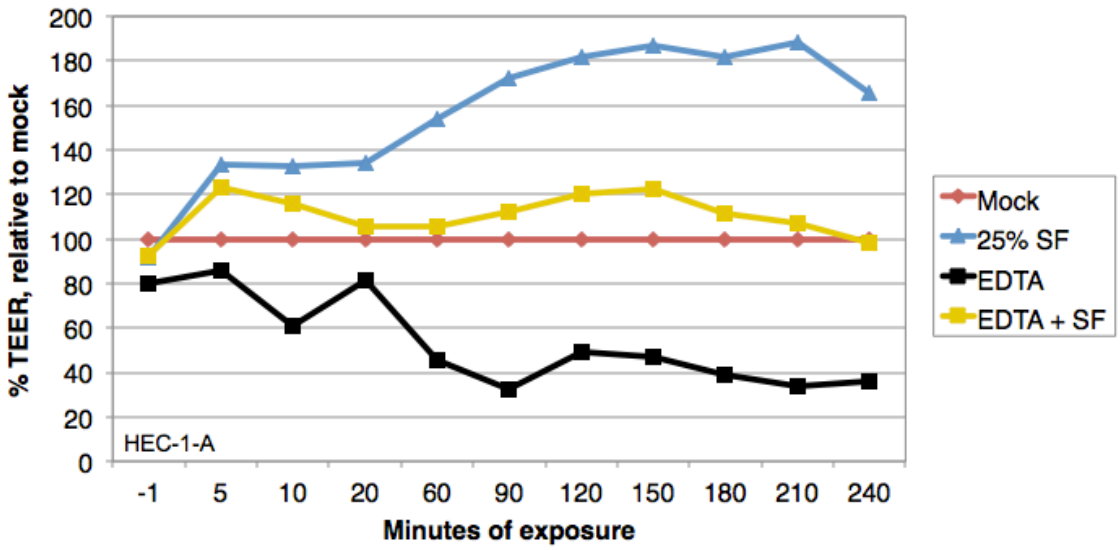
% TEER, relative to mock
200
180
160
140
120
100
80
60
40
20
0
HEC-1-A
-1
5
10
20
60
90
120
150
180
210
240
Minutes of exposure
Mock
25% SF
EDTA
EDTA + SF

Figure A2.7B

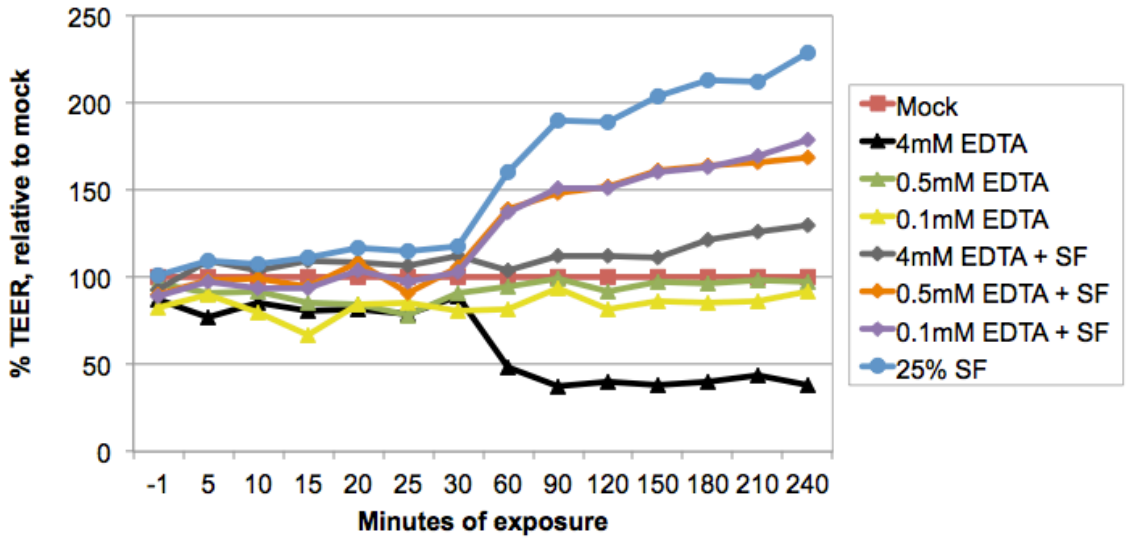

Figure A2.7 EDTA abrogates the effects of SF on resistance. (A) Transwells incubated in a combination of 4mM EDTA and 25% SF resulted in an abrogation of the effects of SF on resistance to nearly mock level. (B) The dampening effect of EDTA on SF was dose-dependent.

A2.4.9 SF restores TEER when epithelial tight junctions have been broken

In order to bring these findings into a physiological context, it was necessary to determine if SF was able to increase TEER in a scenario in which the monolayer had already been compromised. Monolayers were pre-exposed for 30 min to EDTA, which resulted in a decrease in TEER down to 30% relative to mock transwells (Fig. A2.8A). After removal of EDTA, SF was added to the wells and changes in resistance were observed over the course of 5 h. By 90 min, resistance had recovered to mock level and further increased until the end time point, peaking at a 205% increase (Fig. A2.8A). In order to determine whether the natural recovery process of the tight junctions augmented the semen-induced restoration of TEER following removal of EDTA, HEC-1-A monolayers were pre-exposed to EDTA and then monitored for tight junction reformation in fresh media alone. By 1 h the epithelial cells had already started to recover to 65%, and by 4 h, resistance had almost returned to mock level at 89% (Fig. A2.8B). This indicates that the changes induced by EDTA were not permanent, suggesting that in a scenario in which the FRT epithelium has been damaged at the level of tight junctions prior to intercourse, semen may be able to reverse the detrimental effects and speed tight junction resealing, potentially reducing transmission of STD pathogens, or preventing the loss of sperm during passage through the FRT in the context of reproduction [156].

Figure A2.8A

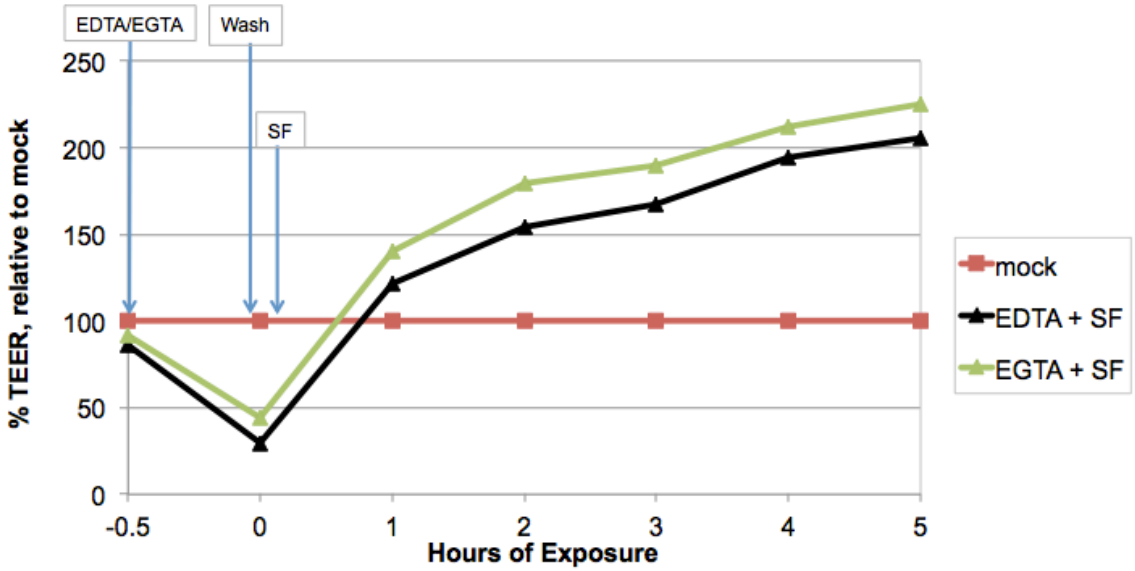
EDTA/EGTA
Wash
SF
250
200
150
100
50
0
% TEER, relative to mock
-0.5
0
1
2
3
4
5
Hours of Exposure
mock
EDTA + SF
EGTA + SF

Figure A2.8B

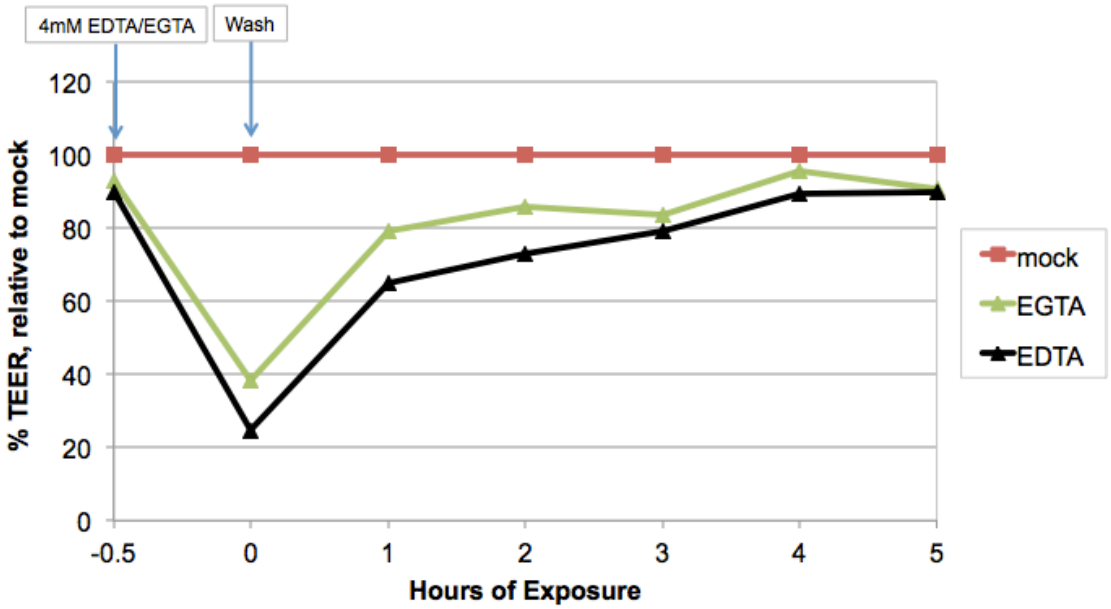

Figure A2.8 SF restores tight junction resistance in compromised monolayers (A) Transwells were pre-incubated in EDTA or EGTA for 30 min to break down tight junctions. After EDTA and EGTA were replaced with SF. (B) Although monolayers will gradually reseal tight junctions on their own after EDTA/EGTA removal, the process is slow--barely reaching mock levels (85%) by 5 h.

TEER is an indirect measure of tight junctions, therefore, in order to correlate changes in resistance with changes in tight junctions proteins, the monolayers were visualized by immunofluorescence staining for zonula occludin-1 (ZO-1), a scaffolding protein that acts as an accessory to the transmembrane tight junction protein complexes of claudins and occludins at the cell surface [177]. Differences in healthy monolayers and semen-exposed monolayers were difficult

to distinguish as both monolayers highly expressed ZO-1 (Fig. A2.9AB), which resonates with the subtle changes in permeability observed previously (Fig. A2.3). Visually, the changes between an intact, relatively impermeable mock monolayer (350 Ω) were indistinct compared to an even less permeable monolayer post-SF exposure (540 Ω). An analogous problem was encountered in a similar study in which slight changes in resistance were inconspicuous across intact epidermal keratinocyte monolayers after staining with occludin and ZO-1 during TLR2 stimulation [159]. Therefore, to gain a better contrast the EDTA-only exposed HEC-1-A monolayers were directly compared to the “recovery” monolayers that had been pre-incubated with EDTA followed by SF incubation. While mock exposed wells had a distinct pattern that is characteristic of ZO-1 staining around the perimeter of cells [159], just 30 min of EDTA exposure was enough to result in a profound absence of this network, manifesting as a punctate staining pattern (Fig. A2.9C). However, incubation of EDTA-pre-exposed monolayers in SF for 4 h resulted in a return of the ZO-1 network that was visually similar to mock monolayers (Fig. A2.9D).

Figure A2.9A

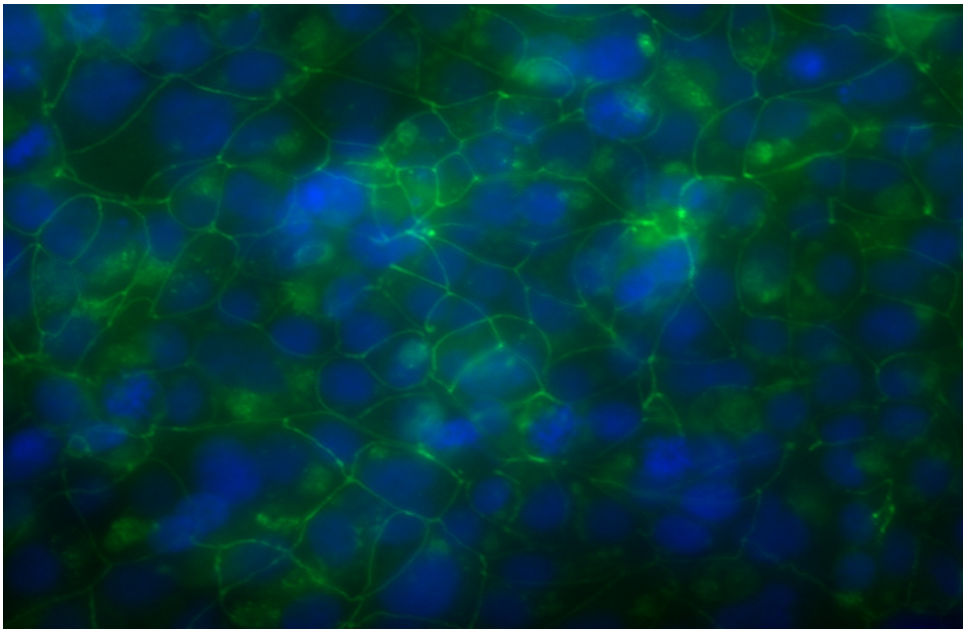

Figure A2.9B

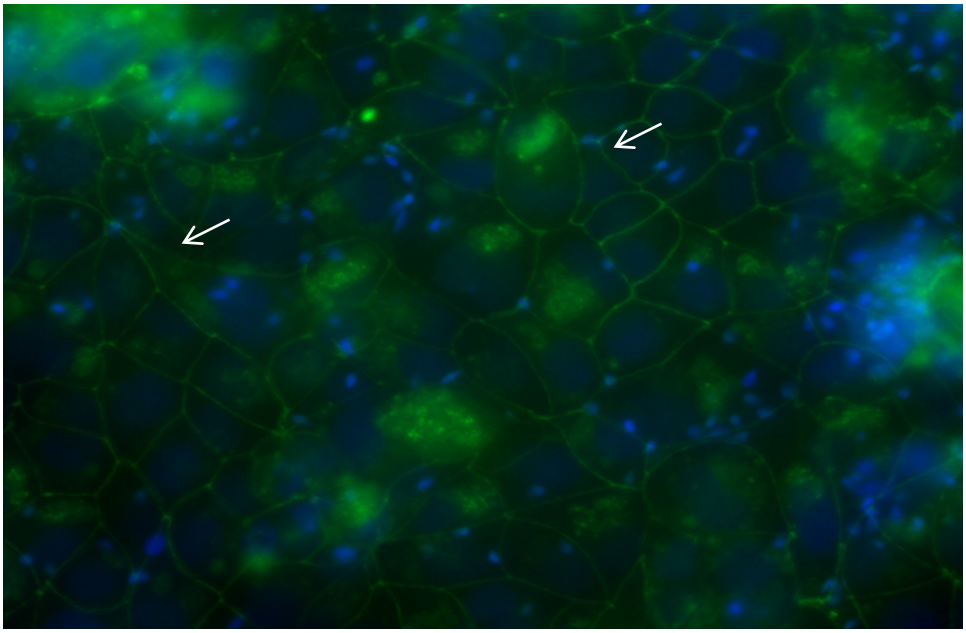

Figure A2.9C

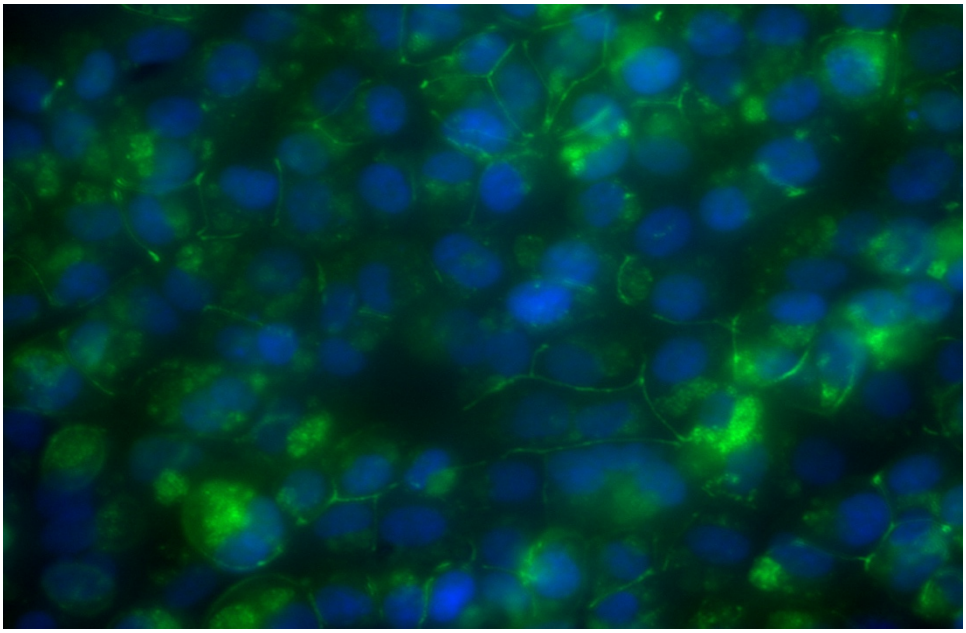

Figure A2.9D

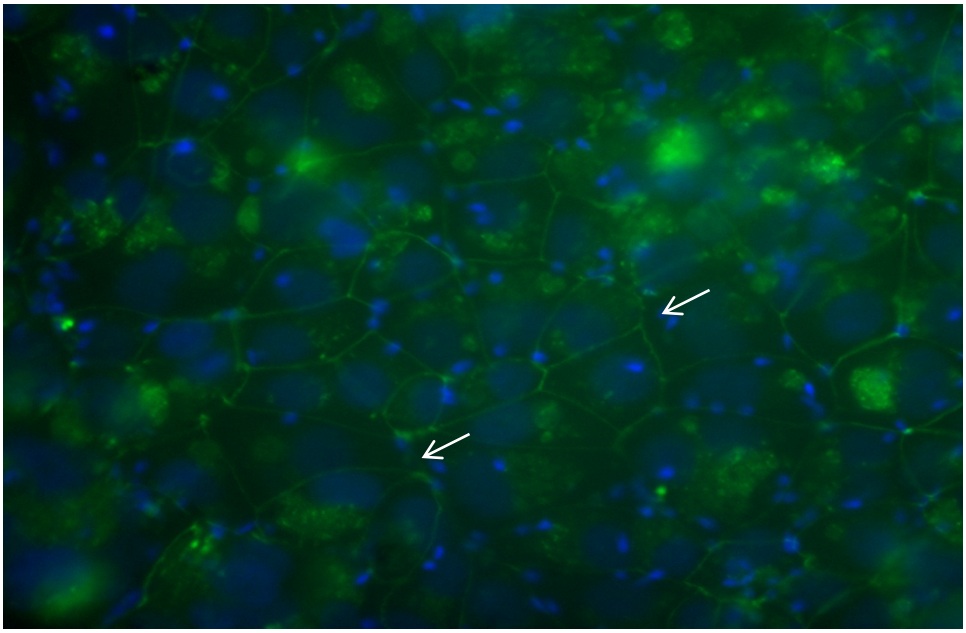

Figure A2.9 Subtle changes in ZO-1 staining are not detectable visually among mock and semen exposed monolayers. HEC-1-A cells incubated apically with 25% SF were stained in transwells for ZO-1 protein expression using a FITC conjugated antibody. The high levels of resistance in mock monolayers (A), about 350 Ω, corresponded to a high degree of ZO-1 staining around the perimeter of the cells. Interestingly, SF-exposed monolayers (B) also demonstrated an impressive staining for the ZO-1 network that was virtually indistinguishable from the mock monolayers, despite their high resistance readings of over 500 Ω. In order to make a more striking contrast, the staining patterns of monolayers exposed to EDTA for 30 min (C) were compared to monolayers that had been pre-incubated with EDTA for 30 min, followed by SF for 4 h (D) in order to assess the degree of recovery that occurred in the presence of SF. 60X magnification. Blue, DAPI/nucleus; green, FITC/ZO-1. White arrows highlight small blue dots that are examples of DAPI stain taken up by the acrosome of spermatozoa, which remained despite vigorous washing of the monolayers.

A2.4.10 Calcium in seminal fluid is partially responsible for increasing TEER

EDTA is a non-specific chelator that induces the break down of tight junctions by removing metal cations from the environment. Since SF is capable of overcoming this effect, it was hypothesized that the factor responsible for increasing resistance may be one of the several metal cations contained in SF. Cells were exposed to physiological concentrations of $MgCl_2$, $CaCl_2$, KCl, and $ZnCl_2$ [32] in order to determine if they contributed to the effects on TEER. Upon exposure, $MgCl_2$, $CaCl_2$, and KCl induced a partial increase in TEER—123%, 119%, and 113%, respectively—as compared to the 160% increase induced by 25% SF (Fig. A2.10A). However, not all metal ions were able to alter resistance, as the $ZnCl_2$ exposed monolayers remained at mock level (data not shown).

In order to determine the effect of removing cations from semen in a more specific manner, the same experiment was repeated, substituting EDTA for EGTA, which preferentially chelates calcium ions from the environment. EGTA acted similarly to EDTA, mediating a decrease in resistance comparable in magnitude and time (Fig. A2.10B, and Figures 8) and abrogating the effects of SF on TEER to a similar degree (Fig. A2.10B). These studies demonstrate the contribution of semen-derived metal ions to the observed increases on resistance, but specifically highlight the role of calcium, since EGTA chelation of predominantly calcium ions from the environment resulted in a dramatic decrease in resistance whereas the addition of calcium partially restored TEER relative to SF. Considering that seminal fluid is able to induce recovery of resistance in epithelial monolayers compromised

Figure A2.10A

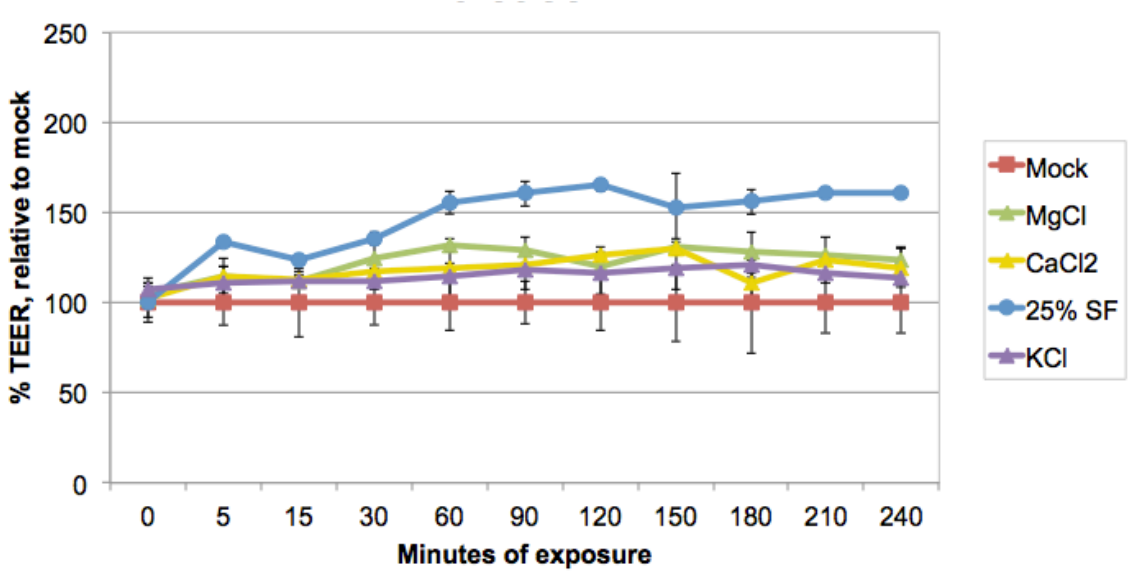

Figure A2.10B

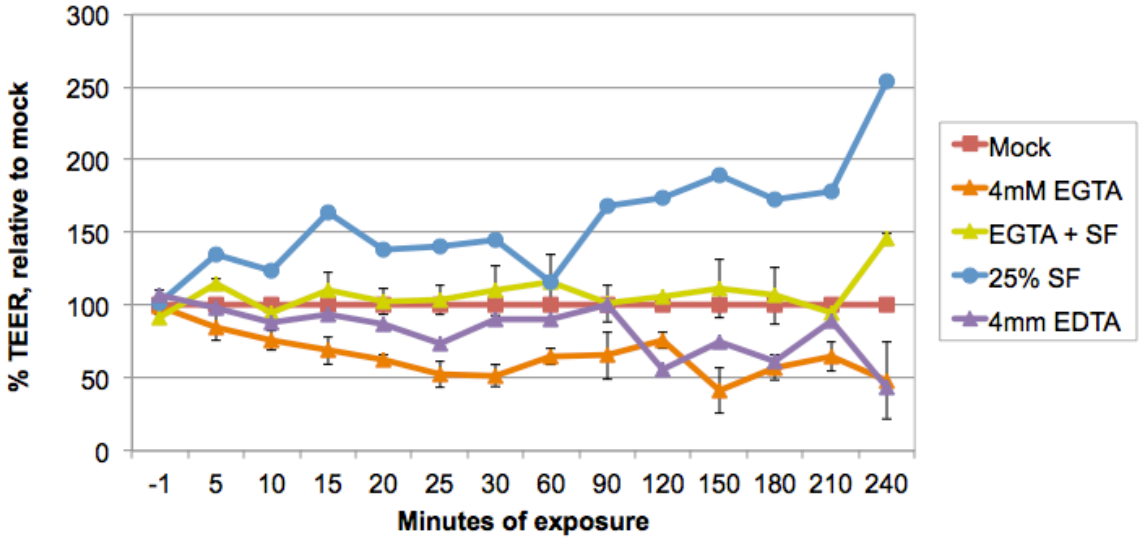

Figure A2.10 Chelation of cations results in a reduction of TEER in semen exposed monolayers. (A) HEC-1-A monolayers incubated apically with physiological concentrations of $CaCl_2$, KCl, and $MgCl_2$ present in seminal fluid resulted in a partial increase in resistance relative to whole semen. (B) HEC-1-A monolayers incubated apically with EGTA, a preferential chelator of calcium, resulted in a time-dependent decrease in resistance similar in magnitude to EDTA.

with either EDTA or EGTA (Fig. A2.8 and 10B), the next step was to determine whether the post-damage-recovery mechanism had specificity regarding the type of damage present. Like EDTA and EGTA, mannitol is well characterized for its ability to break down tight junctions, but does so by an alternative mechanism in which an osmostic imbalance is produced, causing the cells in a monolayer to shrink and physically separate the tight junction strands [198]. In this scenario the mannitol mimics a monolayer compromised by a microabrasion. As expected, a 30min pre-exposure to mannitol decreased resistance in HEC-1-A cells in a time-dependent manner down to 54% relative to mock; however, simultaneous exposure with SF did not result in an increase in resistance above mock levels (data not shown). Of note, the slight recovery evident beginning at 30 min post-SF-replacement was not due to the effects of factors in semen, but was attributed to the natural recovery of the osmotic balance in the cells after receiving fresh media leading to tight junction resealing (data not shown). Furthermore, when HEC-1-A monolayers were incubated simultaneously with 25% SF and 0.1% C31G, a surfactant known to break up the cell membrane [199], seminal fluid was not able to overcome the damage caused by C31G, but it did however reduce the rate at which resistance dropped (data not shown). These findings suggest that the mechanism by which SF induces changes in resistance is attributable to pathways related specifically to tight junctions and therefore does not protect from all physical damaging agents.

A2.4.11 Heat denaturation of proteins contained in SF results in a partial decrease in TEER

The increased resistance is in part mediated by calcium contained in semen (Fig. A2.10A), implicating the contribution of an additional unidentified factor that may be responsible for producing the remainder of the partial increase. SF is complex in composition and contains many constituents in addition to metal ions, including cytokines, chemokines, and growth factors. These soluble factors are located in the seminal plasma, which was shown to be the active fraction of SF (Fig. A2.4). To determine whether a soluble factor in the SP was contributing to the increased resistance, SF was heat inactivated to denature the proteins. Regardless of heat inactivation method used, denaturation resulted in a repeatable and significant partial reduction in TEER (Fig. A2.11A). This suggests that although the addition of calcium alone is able to induce a partial increase in resistance, a heat-sensitive, soluble SP-factor may be responsible for inducing the remainder of the observed increase. Using this information, the cells were simultaneously exposed to heat-inactivated SF in the presence of EGTA in order to both inactivate the soluble factors and remove the calcium component of SF. However, despite these steps, there was only a 2/3 reduction in resistance rather than full inhibition of the semen-induced increase, implicating an as yet unidentified third component contributing to this effect (Fig. A2.11B).

Figure A2.11A

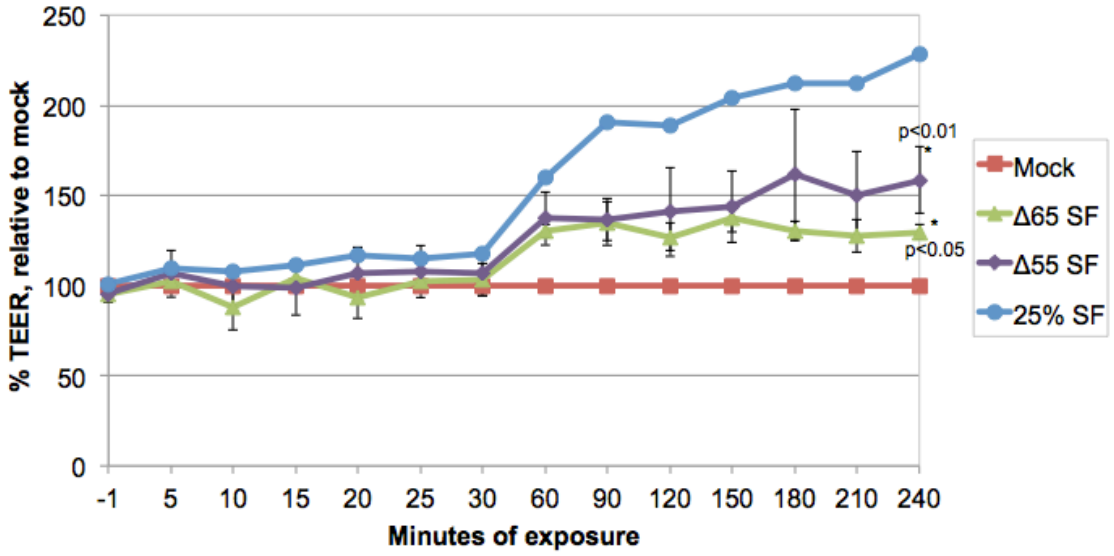

Figure A2.11B

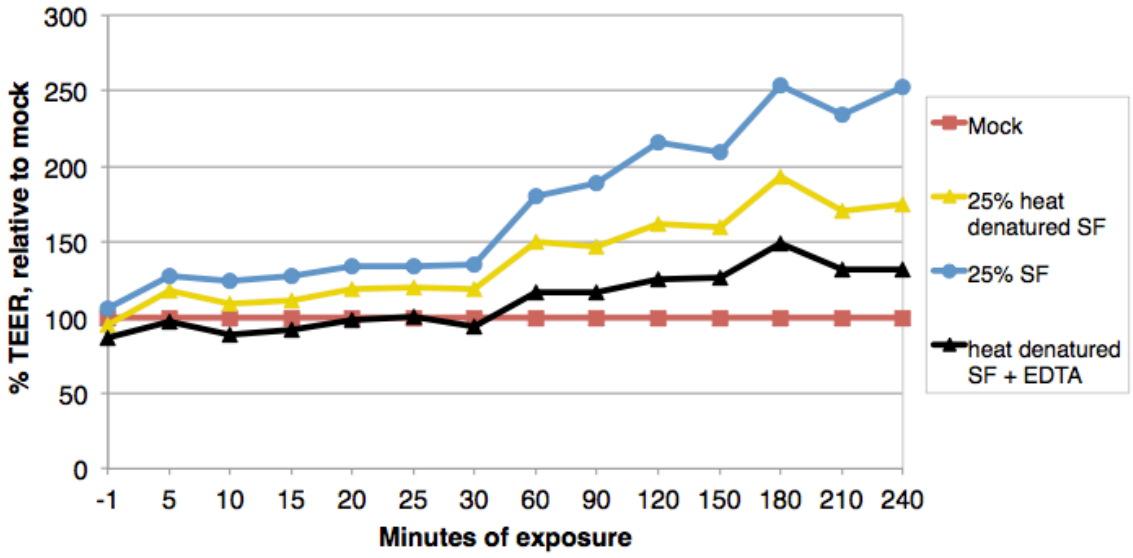

Figure A2.11 Heat denaturation of proteins and chelation of multiple metal ions in SF results in a decrease in SF-induced effects on resistance. (A) Incubating HEC-1-A monolayers with heat denatured SF resulted in a partial increase in resistance relative to 25% SF. (B) Heat inactivated SF was combined with 4 mM EDTA, which removes cationic metal ions, resulted in an increase in resistance of only 1/3 that of the whole SF.

A2.4.12 SF from healthy donors is slightly pro-inflammatory in cytokine content

To determine the identity of the heat-labile soluble component, seminal fluid was screened for 30 protein analytes in a luminex assay. The purpose of identifying semen content was to lead to clues as to what factor or combination of factors may be responsible for augmenting TEER. Although the luminex methodology and results are described in detail in Appendix Chapter 1, what is important to note here is that SF contained very high concentrations of growth factors, chemokines, and pro-inflammatory cytokines. Based on that analysis and information contained in literature, lead factors were identified and evaluated individually at physiologically relevant semen concentrations for effects on TEER. One such factor was epithelial growth factor (EGF), which is present at a high concentration of 2867.1 pg/ml in 25% concentrated SF and has been shown to be involved in tight junction formation [200]. However, when HEC-1-A transwells were incubated with 2 ng/ml of EGF, there were no changes in resistance (data not shown). Similarly, when prostaglandin E_2 (PGE_2), another concentrated semen growth factor, was evaluated at the physiological concentration of 16,000 pg/ml, there were also no effects on resistance (data not shown).

Searching the literature for information on prominent reproductive factors contained in seminal fluid, one protein often discussed is transforming growth factor-β (TGF-β). Although this factor was not included in the 30-plex luminex screen, it is present in seminal fluid at the highest concentration of anywhere else in the human body—other than breast milk—and likely plays a key role in

protecting the fetus from clearance by suppressing the local maternal immune response in the FRT [70]. Of particular relevance are cancer studies on epithelial-to-mesenchymal transition (EMT), which indicate that TGF-β may have concentration-dependent effects on tight junctions [201, 202]. Based on this information, it was possible that the high concentrations of TGF-β present in seminal fluid were contributing to the increased resistance. To address this, HEC-1-A and End1 monolayers were exposed in transwells to either a low or high (physiological) concentration of TGF-β for 4 h (data not shown). However, in this model, TGF-β did not have any effects on the epithelium when evaluated for changes in resistance, permeability, or cell viability, suggesting that this factor is not responsible for the rapid, increased resistance observed during short-term SF exposure (data not shown). Additional experiments will be required to determine whether TGF-β is able to overcome tight junction compromise in epithelial cells as opposed to playing a role in increases in resistance on already intact monolayers.

Using the transwell model, it is possible to replicate events that naturally occur during intercourse that may contribute to changes in resistance. TGF-β is expressed in the latent form in seminal fluid and requires activation; one possible mechanism of activation may be acid-activation within the FRT [70]. The vaginal tract maintains a low pH of 4. However, when alkaline semen at pH 7.2 [174] is introduced to the acidic environment of the vagina, there is a neutralization process that occurs, resulting in a pH transition. It is important to determine whether TGF-β activation in semen could affect the magnitude of SF-induced effects on resistance. To study the contribution of pH and TGF-β activation on tight junctions

Figure A2.12A

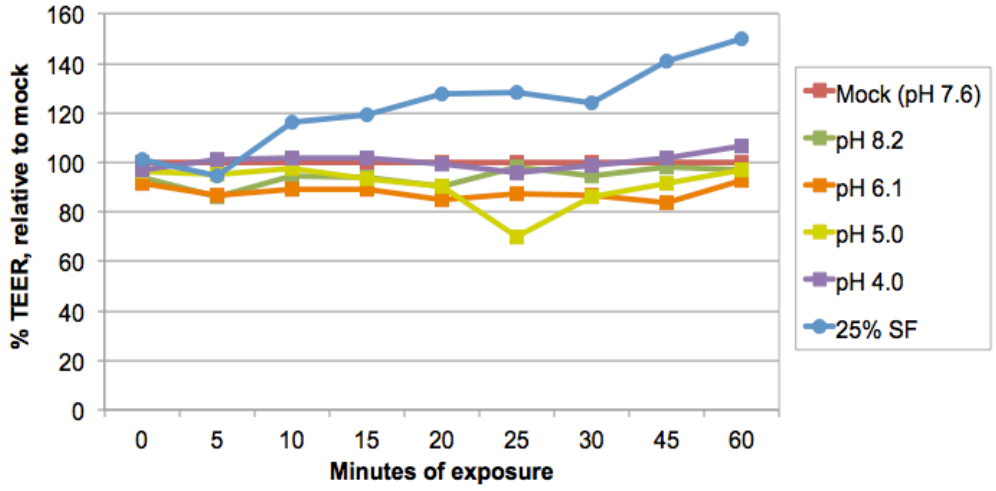

Figure A2.12B

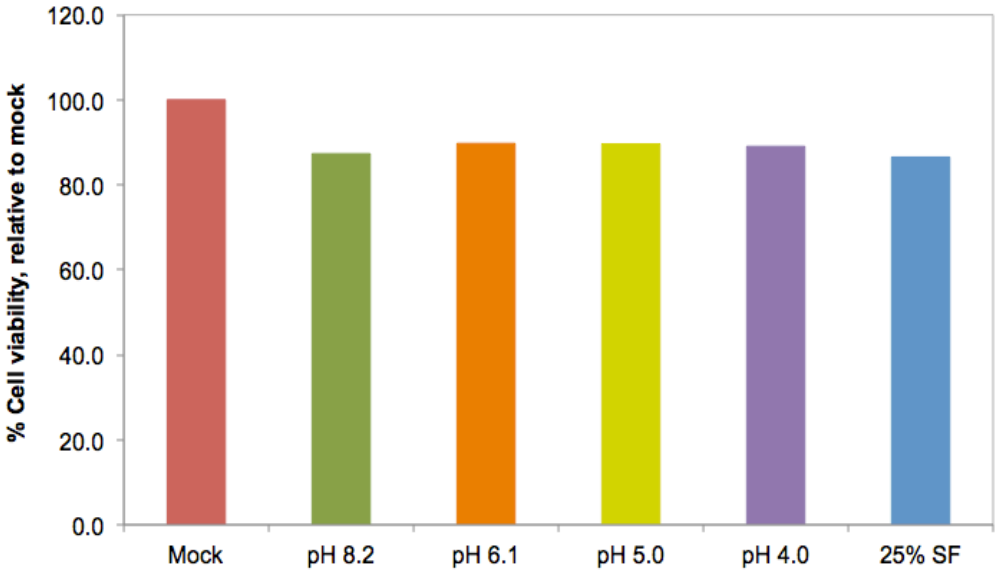

Figure A2.12C

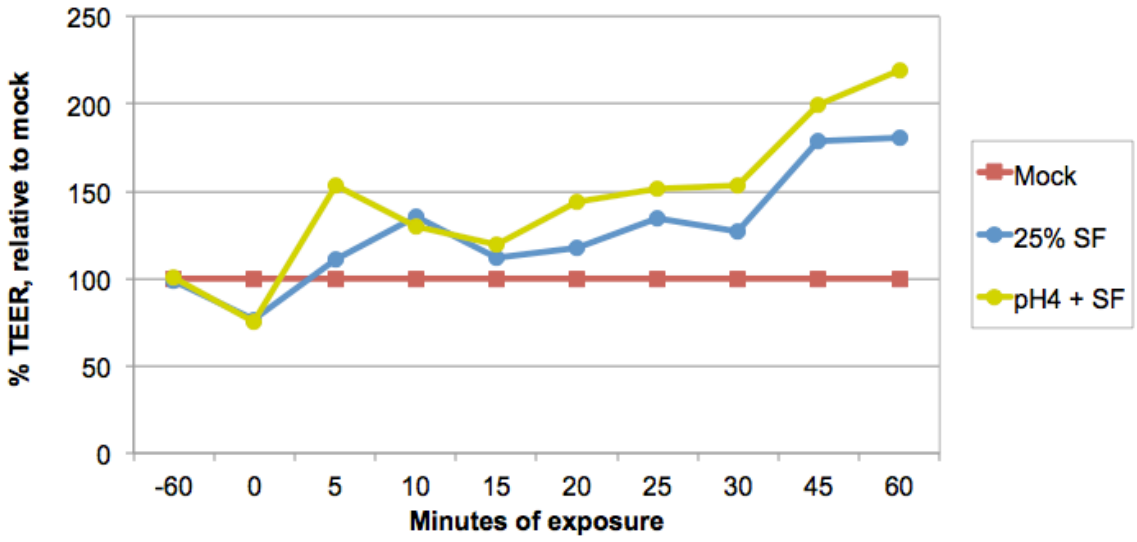

Figure A2.12 Changes in pH do not alter resistance or affect cell viability but the pH transition may slightly increase the magnitude of SF-induced effects on resistance. HEC-1-A monolayers were incubated in media pH concentrations ranging from alkaline, pH 8.2, to acidic, pH 4.0, for 1 h. (A) No changes in resistance were induced when cells were cultured with high or low pH. (B) pH did not have a detrimental effect on cell viability at the time points observed. (C) Ect1 monolayers were cultured for 1 h in media titrated to pH 4 in order to replicate the acidic environment of the vagina. SF was then added to the apical chamber, mimicking the pH transition phase of intercourse in which alkaline SF is deposited into the vagina, inducing a neutralization of pH in the local environment.

in a realistic configuration, a model of the pH transition that occurs during intercourse was developed. Culture of HEC-1-A and Ect1 monolayers in a range of acidic, neutral, and alkaline media conditions for 1 h alone was not enough to induce changes in resistance (Fig. A2.12A) or cell viability, with only a 13% decrease at the most alkaline pH of 8.2 (Fig. A2.12B, Ect1 data not shown). Once it was established that the cells could survive culture in low pH, Ect1 monolayers were pre-incubated for 1 h in pH 4 media in order to replicate the acidic environment of the vagina, followed by the addition of semen to induce a pH transition. Interestingly, the peak of SF-induced resistance after 4 h was slightly higher in monolayers cultured at a low pH when compared to SF exposure in neutral pH culture medium (219% and 180%, respectively) (Fig. A2.12C); further study will be required to determine if this difference is significant. Using this model, it was possible to determine that although pH alone does not affect resistance, there is some indication that factors, perhaps TGF-β, in seminal fluid may further alter activity once *in vivo* and modify tight junction expression.

A2.4.12 SF interacts with apically expressed surface molecules to induce changes in TEER

During the pursuit to identify the unknown soluble semen factor, it was hypothesized that there may be an interaction between the unknown factor and a receptor on the surface of epithelial cells which initiates tight junction regulating pathways. The preliminary data regarding tissue specificity indicates that a specific receptor may be involved since non-FRT tight junction expressing cells did not respond to semen. To determine if the effects of SF were mediated through a

surface receptor or through other methods of tight junction regulation, the transwell cell cultures were incubated with 25% SF in the basolateral chamber. Many factors have surface specificity in the context of TEER and transwells, such as bone morphogenetic proteins (BMPs), which interact with BMP receptors (BMPRs) located exclusively on the basolateral surface of the polarized epithelium to increase TEER [203]. As a control, monolayers were incubated basolaterally with 4 mM EDTA or EGTA, which resulted in a decrease in resistance similar to the rate and magnitude of apically exposed cells (Fig. A2.13A, EGTA data not shown). However, when 25% SF was incubated in the basolateral chamber, no significant changes in resistance were observed in either HEC-1-A or Ect1 cells (Fig. A2.13B, Ect1 data not shown). Interestingly, apical SF was unable to overcome the effects of basolateral EDTA during simultaneous exposure. This particular observation may have an important implication in the context of male-to-female STD pathogen transmission in circumstances where the FRT is vulnerable due to existing damage prior to SF exposure, possibly from an existing infection or inflammation associated with menstruation [204] (Fig. A2.13C). In summary, the data here suggest that SF interacts in a specific manner with an apically expressed surface receptor in order to induce changes in resistance.

A2.4.13 TLR2 and semen interaction

Previous literature has shown that VK2, Ect1, End1, and HEC-1-A cells express functional Toll-like receptor 2 (TLR2) receptors on the surface [159] [205]. TLR2 is a pattern recognition receptor that recognizes a variety of structural features, including bacterial- and viral-derived molecules, which results in the

Figure A2.13A

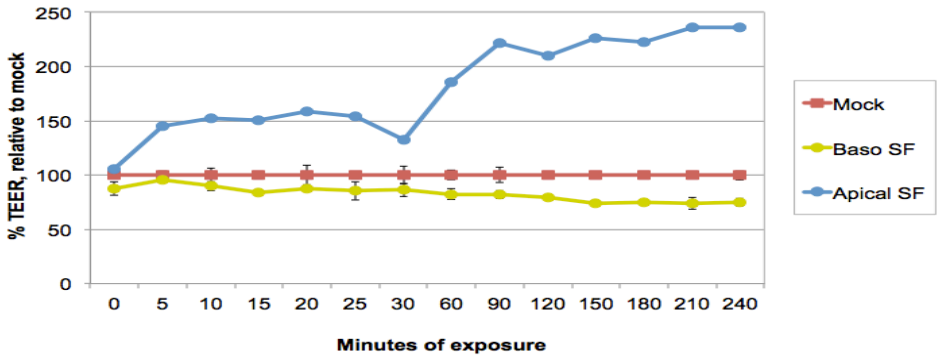

Figure A2.13B

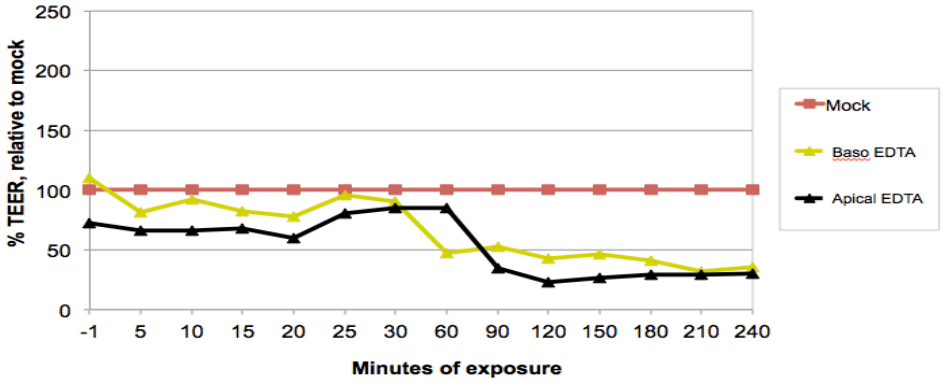

Figure A2.13C

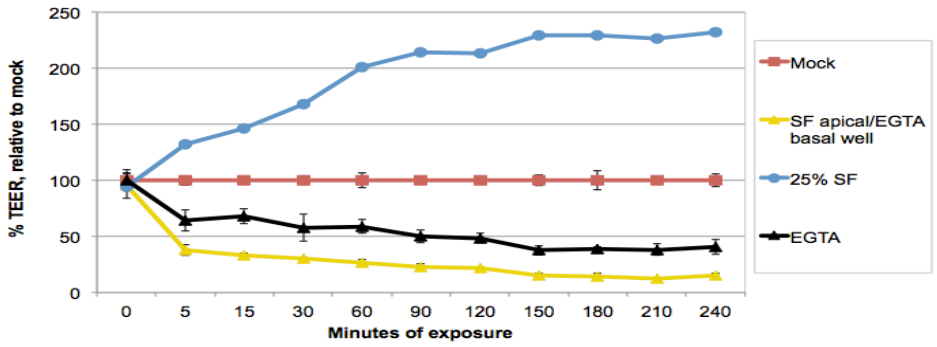

Figure A2.13 SF mediates and increase in TEER in a surface-specific manner. HEC-1-A transwells were incubated in the basolateral chamber with either (A) 4mM EDTA, a control to ensure that the monolayers are capable of responding to exposure from the basal surface, (B) 25% SF, or (C) simultaneous exposure to 25% SF in the apical well and 4mM EGTA in the basolateral well, in order to determine if subepithelial-sourced decreases in resistance can abrogate the effects of SF upon deposition. Although EDTA produced a time-dependent decrease in resistance almost identical to apical EDTA, SF did not affect resistance, with TEER remaining around mock level. Incubation with EGTA (C) in the subepithelial space overcomes the effects that SF mediates during apical exposure, resulting in a rapid and sustained decrease in resistance.

activation of innate immunity [206]. Most recently, gut studies of *Salmonella typhimurium* have demonstrated that the bacterially derived curli amyloid fibrils induce changes in resistance via interaction with TLR2 on T84 gut cells [206]. This is especially relevant in light of the recent discovery of SEVI (Semen-derived enhancer of virus infection) in seminal fluid, a conglomeration of PAP-derived peptide fragments naturally present in semen that aggregate to form cationic amyloid fibrils [142]. Studies suggest that this fibril may be involved in increasing HIV-1 transmission via electrostatic interactions with the virus, neutralizing virion-host cell surface charge repulsions and thus facilitating infection [142, 143]. Denaturation of SEVI may alter conformation and neutralize the enhancing activity [143]. Based on this information, we hypothesized that the unidentified heat-labile factor present in SF may induce changes in resistance via TLR2 interaction. HEC-1-A, Ect1, and VK2 monolayers pre-incubated with an anti-TLR2 blocking antibody were exposed to SF and monitored for changes in TEER. The results revealed that a TLR2-semen interaction was not involved, as blocking of the receptor did not dampen the SF-mediated increase in TEER (Fig. A2.14, Ect1 and VK2 data not shown). The ability of the cells to respond to TLR2 stimulation was confirmed by incubation with a positive-control, peptidoglycan (PGN), which produced a rapid increase in resistance that persisted out to the later time points, but was abrogated in the presence of the TLR2 antibody (data not shown). While further study will be necessary to eliminate a possible role for TLR2 interactions at time points beyond

Figure A2.14

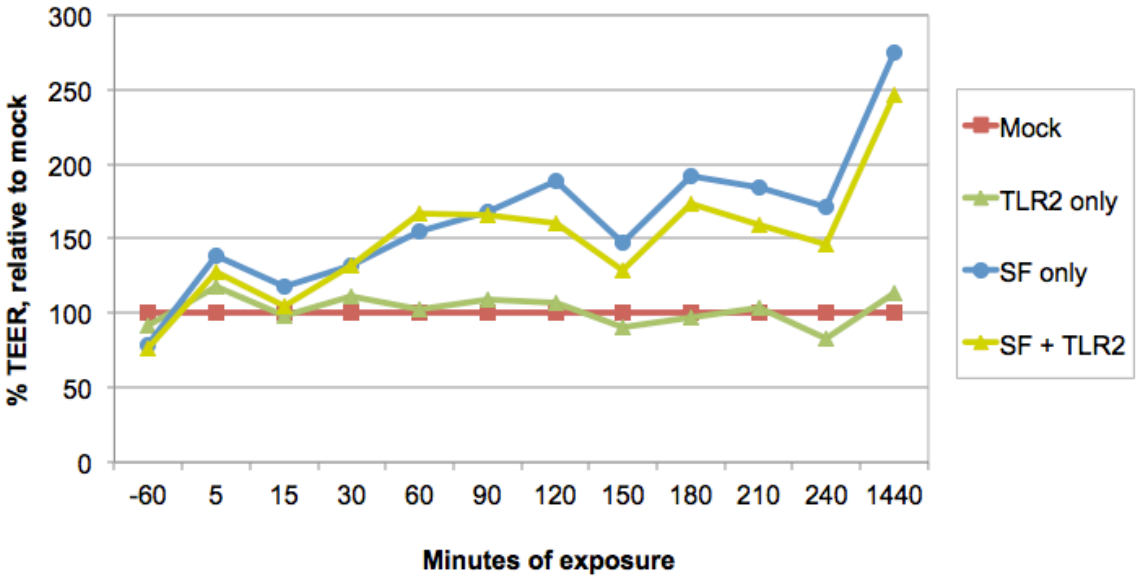

Figure A2.14 SF does not mediate changes in resistance through an interaction with TLR2. Pre-incubation of HEC-1-A monolayers with a neutralizing antibody 1 h prior to 25% SF exposure did not alter the effects of SF on resistance even out to 24 h of continuous exposure. Antibody alone did not affect normal resistance readings.

4 h, the factors involved in the initial rapid, increase in resistance observed within 5 min out to 4 h of SF exposure remain elusive.

A2.4.14 SF-induced effects on resistance may protect against sexual transmission of pathogens

In addition to reproductive implications, the changes induced by SF are also important when considered in the context of disease transmission. The decrease in permeability following SF incubation may have a secondary effect of protecting against sexual HIV-1 transmission by decreasing paracellular passage of the virus. Previous studies have indicated that HIV-1 alone has a detrimental effect on TEER, with exposure resulting in a consistent decrease in resistance across genital epithelial cells regardless of incubation time, concentration of virus, or strain of virus used [191]. Furthermore, the proposed mechanism of decrease was attributed to a gp120 interaction with the epithelial cell surface, resulting in a rapid breakdown of tight junctions [191]. However, this study on HIV-1 transmission was performed in the absence of seminal fluid, which itself will contribute to effects by modulating the local resistance and immune responses and may possibly interfere with the proposed virus-epithelial interaction. To study the effect of transmission across the epithelium in the context of seminal fluid, HEC-1-A, End1, and Ect1 monolayers were incubated apically with either HIV-1 BaL only or HIV-1 BaL mixed with SF. During incubation no effects on resistance were observed on TEER in the virus-only control out to 4 h, nor was there a change in the degree of increase induced by SF in the presence of the virus (Fig. A2.15A-C). The stability of

Figure A2.15A

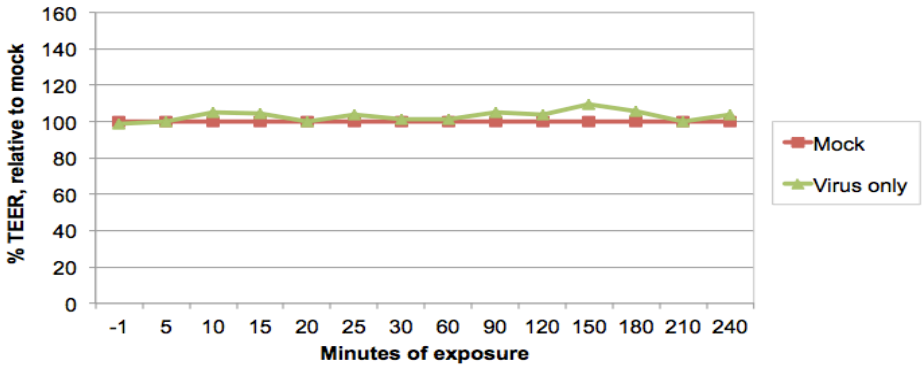

Figure A2.15B

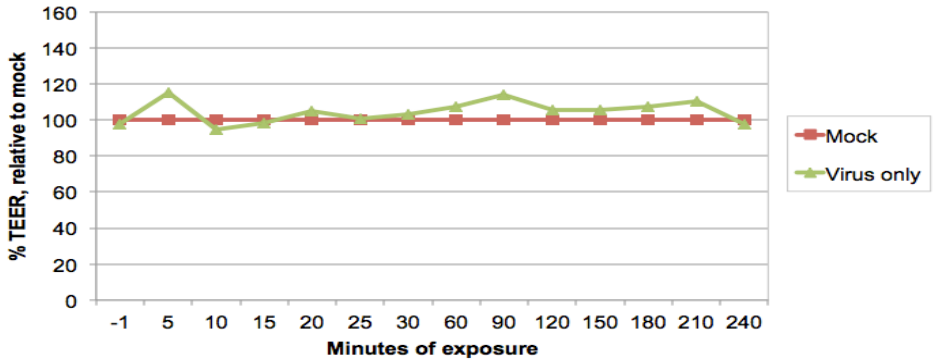

Figure A2.15C

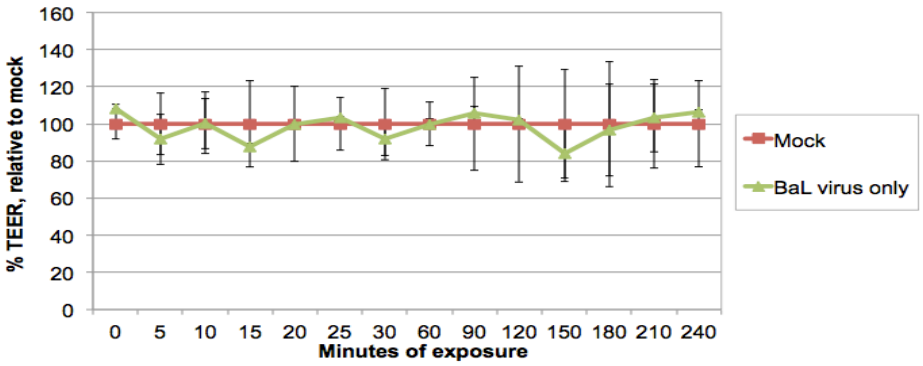

Figure A2.15D

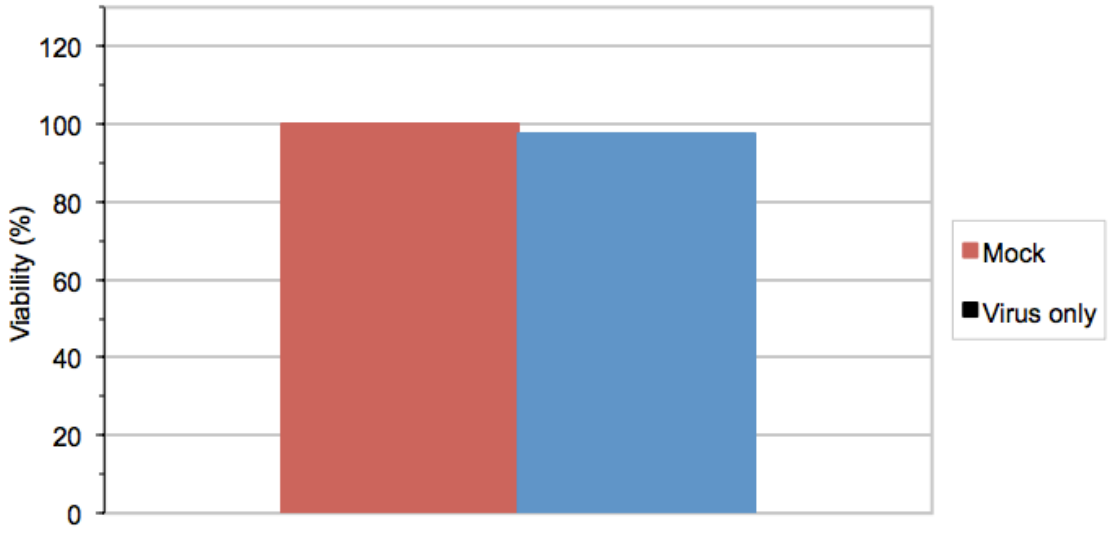

Figure A2.15 HIV-1 BaL does not induce changes in resistance or viability in reproductive monolayers. (A) HEC-1-A, (B) End1, and (C) Ect1 monolayers were incubated apically for 4 h with HIV-1 BaL and then assessed for changes in TEER either with virus alone, or virus in the presence of 25% SF. HIV-1 BaL alone did not induce a change in resistance. (D) In order to rule out any external effects as a result of decreased viability in the presence of virus, the monolayers were assessed via MTT assay. No detrimental effects induced by HIV-1 BaL were observed at the concentrations or time points utilized (End1 pictured here, HEC-1-A and Ect1 data not shown).

resistance was also consistent with the viability assay, demonstrating that the virus did not induce cell death in these experiments (Fig. A2.15D). To determine whether increased resistance corresponded to a decrease in viral penetration into the basolateral chamber, the media from the basolateral chamber of each transwell was incubated with P4R5 cells. In this model, if even a single virus migrated across the epithelium (which would be undetectable by p24 assay) the virus could amplify through multiple rounds of infection in the P4R5 cells resulting in a proportional β-galactosidase (β-gal) output. Wells in which the virus had been co-incubated with SF resulted in a 50% reduction in β-gal output relative to wells that had virus only (Fig. A2.16). Further study will be required to determine if this effect is significant, along with follow-up using a more precise method for quantifying virus migration across the epithelium. The preliminary studies presented here implicate a protective role for semen from healthy donors by decreasing permeability, thereby potentially reducing HIV-1 transmission.

A2.5 Discussion

Recent studies have only begun to shift the view of seminal fluid as a carrier of sperm to an active immunological participant in the FRT, implicating the active role it may play in both reproduction and STI susceptibility. The studies presented here reveal that in addition to the previously documented immunological effects, SF also mediates direct changes on the epithelium at the level of tight junctions, the initial step that drives subsequent local environmental processes. Physiological parameters based on previous work were utilized in order to develop a model of

heterosexual intercourse and study these changes in a relevant and accurate context. Our initial observations indicate that semen induces an increase in resistance in the epithelial monolayer that is both dose- and time-dependent. Although, the concentration of SF that ultimately occurs in the FRT is debatable, we found that regardless of degree of dilution, these effects persisted across concentrations ranging from 50% to as low as 0.1%. However, the most interesting observation was that the increase in resistance that SF induced occurred very rapidly, with significantly elevated TEER evident by 5 min of exposure, suggesting that this process is likely independent of tight junction protein production. At longer time points, though, it is possible that protein production is a contributing factor in order to sustain the continued increase in resistance that eventually peaks beyond 4 h.

It is interesting to note that the changes that SF induces are not permanent, as removal of semen in a manner that corresponds to the time frame of clearance from the FRT during coitus resulted in a gradual decline in resistance, eventually resuming back mock level after 4 h and remaining stable. Previously published studies indicate that after semen deposition in the reproductive tract, SF is no longer present in large quantities in the vagina due to clearance by several processes including phagocytosis by neutrophils and macrophages; absorption; leakage out of the tract; and the upward migration towards the uterus [28]. It is important to highlight that although the monolayer resumes tight junction expression to “mock level” this does not imply that the monolayer is vulnerable in this state. The permeability studies and staining demonstrate that the monolayers

Figure A2.16

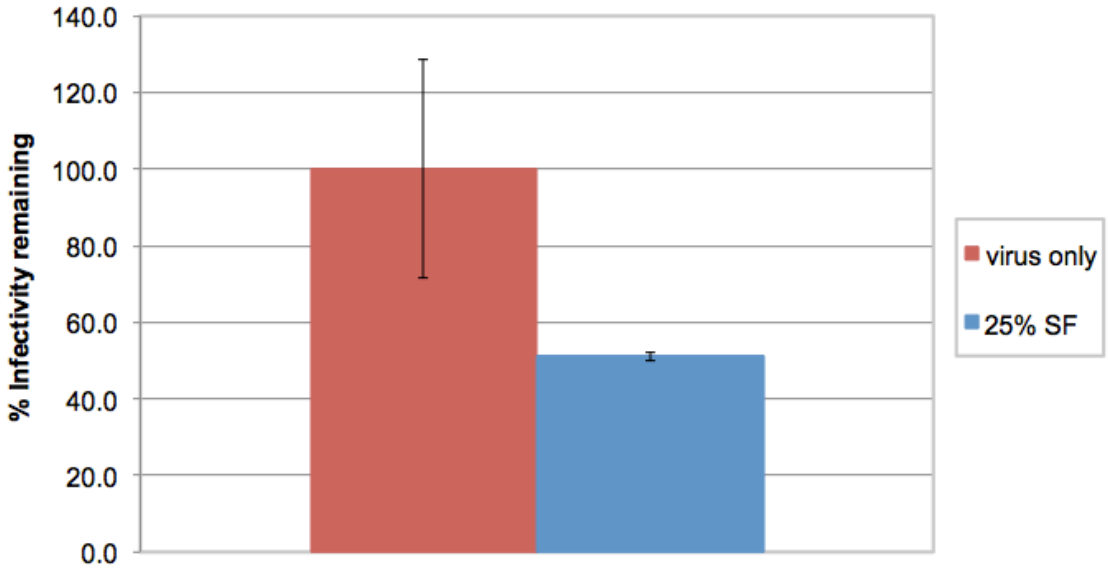

Figure A2.16 SF reduces monolayer permeability to HIV-1. HEC-1-A monolayers were incubated apically with HIV-1 IIIB, a CXCR4 co-receptor utilizing virus that infects more efficiently than HIV-1 BaL. In the presence of 25% SF the level of β-gal was decreased relative to monolayers infected with virus only.

form a relatively impermeable barrier even at baseline levels, which is an innate protective function characteristic of the epithelium lining the FRT. These studies also established that changes in resistance correlated inversely with changes in the monolayer's ability to regulate paracellular passage, proving that increased resistance resulted in a corresponding decrease in permeability. This relationship was corroborated by the staining pattern of the ZO-1 protein network, verifying that the tight junctions remain intact during SF exposure and are responsible for the elevated resistance. Studies of tight junctions have demonstrated that they are a dynamic system of strands, in a constant motion of opening and closing [177, 181, 207]. To make these rapid changes possible there are vesicles containing tight junction complexes located just beneath the cell surface that are at the ready for upregulation and surface expression [97, 207]. The process of vesicle fusion is governed by several pathways, one of which is calcium-mediated [97]. In addition to vesicle trafficking, calcium signaling pathways have been shown to be a part of tight junction formation and regulation [177]. Calcium signaling is one of the few processes that functions quickly enough to produce the rapid increase in TEER observed at the 5 min time point. In general, when calcium is introduced into the extracellular environment signaling may be activated through the calcium sensing receptor (CSR), a G-protein coupled receptor (GPCR), resulting in the release of intracellular calcium stores from the ER which combines with DAG to activate protein kinase C (PKC) and thus increase vesicle fusion at the cell surface, upregulating tight junctions [97, 158]. The rapid

exocytosis of vesicles containing entire junctional complexes at the cell surface may explain the ability for resistance to increase minutes after SF deposition [158]. Eventually, though, the calcium stores would either be depleted or the gradient across the membrane would re-equilibrate, thus it is likely that a second pathway is also activated, which would be responsible for upregulating tight junction protein production in order to sustain the elevated resistance during the later phases after 4 h and peaking around 24 h.

Our studies indicate that calcium is only one aspect responsible for the increased TEER, as the addition of extracellular calcium at the concentration present in SF only partially increased resistance relative to seminal fluid. A second possible factor may be a heat-labile protein in the seminal plasma, since heat denaturation of SF resulted in a partial decrease in semen-induced effects. Soluble factors were pursued as possible leads in the identification this unknown contributor. However, proteins detected by the Luminex assay at high concentrations that have been known to alter tight junctions were ruled out, such as TGF-β, EGF, and PGE_2. Additional data supports the hypothesis that this unidentified protein is acting through a specific protein-epithelial surface receptor interaction, as evidenced by basolateral exposure studies and the lack of effects in non-FRT, tight junction-expressing cell lines. Although TLR2, a surface receptor implicated in tight junction modulation via bacterial studies, was ruled out, further study will be required to identify both the semen factor and apical receptor involved in these processes.

The preliminary evidence presented here suggests that a third factor may also be involved. Mixing heat denatured SF with EDTA, a process that inactivates the proteins and chelates cationic metal ions in semen, did not completely abolish the increase in TEER, maintaining a 1/3 increase in resistance. Exclusionary experiments ruled out any contribution of physical characteristics of SF, such as viscosity, spermatozoa, pH, or serum effects. A previous study of SF in the reproductive tract has proposed that the changes in resistance may be induced by lipids, which are found in semen as part of both the spermatozoa membrane and in the seminal plasma fraction [156, 208]. Diacylglycerol, which is involved in the tight junction regulating signaling cascade induced by extracellular calcium, is an example of one lipid found in semen that may increase surface tight junction expression by activation of protein kinase C (PKC) and subsequent upregulation of ZO-1 [162, 208]. Another possibility is regulation of tight junctions by lipid-derived hormones, such as testosterone. Studies of prostate epithelium have indicated that low serum testosterone levels may result in decreased tight junction expression leading to prostatic hypertrophy; in the context of healthy SF, testosterone may contribute to the persistent increase in resistance observed despite heat-denaturation and cation removal [209].

Identifying the key changes mediated by SF in the FRT is important because these changes have application to many reproductive and sexual transmission studies. From a reproductive standpoint, SF-induced increases in resistance may facilitate fertilization via closing of the tight junctions between the cells lining the reproductive tract, which would: (i) prevent the spermatozoa from

getting trapped in the paracellular spaces, (ii) prevent clearance by immunological factors in the FRT, and (iii) provide a smooth passage across the epithelial surface towards the egg [156]. However, in the context of disease studies, the same factors that alter the environment during fertility may affect the likelihood of a female becoming infected [172]. Preliminary studies have determined that the concentrations of cytokines present in SF from infected males are dysregulated, varying with factors such as stage of disease and treatment intervention [8, 10, 94]. A thorough analysis of semen content from the infected population in future studies will be a challenge as the variability of these factors from one male to another is likely to be high, depending on overall health, current HIV disease status, age, drug use, etc. Interestingly, virus alone may alter tight junction expression. Previous studies have observed decreases in resistance using multiple concentrations and strains of HIV-1 in the absence of semen [191]. This is in contradiction to the results presented here, in which virus did not affect resistance either alone or in combination with SF. Although the difference in observations may be attributable to differences in experimental conditions, such as the types of cell lines used, what is important to consider is that the composition of seminal fluid from an infected male forming the environment surrounding the virus will be different from the healthy males studied here and likely impact both resistance and permeability. In regards to the other two factors involved, calcium and lipids, studies have shown that HIV-infected males also suffer from both low testosterone [210] and calcium deficiency, which may be attributed to infection and/or ART [211]. With the compounding changes in testosterone, calcium, and

cytokines, the protective effect on resistance mediated by semen from healthy men may be absent or reduced in the case of an infected male.

In conclusion, we identified a possible protective role for semen in the FRT in the context of HIV-1 transmission. Exposure to SF resulted in a rapid and sustained increase in resistance that also produced a concurrent decrease in permeability. Our investigations suggest that these changes may be induced by a combination of semen-derived calcium, a soluble protein component, and a lipid portion that mediate an increase in resistance through a specific interaction with the apical interface of the reproductive epithelium. Further study will be required to identify the specific semen protein factor in healthy seminal fluid responsible for initiating these changes. Establishing the epithelial events that take place after semen deposition will prompt future studies to focus on examining whether these factors or events are dysregulated during exposure to HIV-1 infected seminal fluid. Changes in the profile of factors constituting SF in the MRT of an infected male may alter the effects of semen on tight junctions observed here, thereby enhancing passive permeability of the epithelium to virus, resulting in direct access to target immune cell population located in the subepithelial space. The loss of the protective effect induced by semen in the FRT may be the unaccounted aspect of transmission that has been overlooked thus far, providing one explanation as to how a disease with an estimated low-risk of transmission per sex act could lead to the world epidemic present among heterosexual women today.

SUMMARY AND CAREER GOAL

A biologist and microbiologist with more than twelve years of experience looking to utilize his knowledge, skills, and personality in teaching. An out-of-the-box thinker who can inspire and lead with ease. Eventually leading to a faculty position.

SCIENTIFIC EXPERIENCE AND BACKGROUND

Ph.D. GRADUATE CANDIDATE Drexel University 09/2011 – Present

- Perform research and experimentation in a laboratory setting under the guidance of an advisor
- Perform the tasks and duties of a Teaching Assistant (TA) for undergraduate courses
- Take prerequisite classes
    - Advisor: Dr. Fred Krebs

LAB TECHNICIAN & TA Drexel University 03/2009 – 09/2011

- Oversee prepping for the Department of Biology education labs and TA for microbiology and microbiology for health professionals and biology courses
    - Prep, set up, and break down materials for 3-5 different biological lab courses and all corresponding sections per term
    - Work with faculty to improve labs to better serve the student's ability to grasp complex concepts more readily
    - Delegate tasks to the work study undergraduates

ANALYTICAL MICROBIOLOGIST Campbell's Soup Company 01/2008 – 04/2008

- SOP/GMP compliant facility
- Basic food microbiology principles were applied daily
- Enrichment cultures were created and ordered as necessary
- The BAX and Vitek Identification Systems used for identification of contaminants
- Bioassay development and analysis of pilot plant production of Campbell's goods

EDUCATION

Rutgers University New Brunswick, NJ 09/2003 – 05/2007

- Bachelor of Science, Microbial Biotechnology
- Bachelor of Science, Microbiology
- Minor, Biochemistry

Drexel University Philadelphia, PA 09/2011 – Present

- Ph.D. in Biology

TEACHING EXPERIENCE

Drexel University Philadelphia, PA 03/2009 – 06/2013

- Laboratory Instructor for
  - **Microbiology for Health Professionals**
    - Taught microbiological lab techniques utilized in a clinical setting
    - Explained concepts and observed application of concepts
    - Administered and graded assignments and quizzes
- Teaching Assistant for
  - **Microbiology**
  - **Principles of Molecular Biology**
  - **Cells and Genetics**
  - **Essential Biology**
  - **Microbial Food Safety and Sanitation**
  - **Food Microbiology**
    - Helped teach biological lab techniques
    - Helped explain concepts and observe application of concepts
    - Assisted students with group projects; focusing, planning, and execution
    - Helped administer and grad assignments and quizzes

COMPUTATIONAL SKILLS

- Microsoft Office
- Black Board Learning
- Kaleidograph
- DNA Star
- Sigma Plot
- Chromas
- MegAlign
- SeqBuilder
- InterproScan
- Prosite
- WHAT_IF
- ClustalW
- InsightII
- UNIX System
- FlowJo
- R

LAB TECHNIQUES

- PCR
- qPCR
- Western blotting
- ELISAs
- Transfections
- RT-PCR
- Cloning
- Flow Cytometry
- DNA/RNA extractions
- Next Generation Sequencing (Illumina NextSeq)
- Cell culture techniques (Viral, Fungal, Bacterial, and Protozoan)
- Electrophoretic Mobility Shift Assay
- CHiP
- Assay Development
- Fluorescence Microscopy
- BAX/Vitek Identification
- Luminex Assay
- NanoDrop
- Transepithelial Electrical Resistance (TEER)

BIBLIOGRAPHY

Published/In-Press Full-Length Papers (*peer-reviewed)

*1. Keogan, Shawn, **Karl Siegert**, Brian Wigdahl, and Fred Krebs. *Immunomodulation by Seminal Factors and Implications for Male-to-Female HIV-1 Transmission.* Journal of Acquired Immune Deficiency Syndrome. Published.

*2. Keogan, Shawn and **Karl Siegert**, Brian Wigdahl, and Fred Krebs. *Variability in Human Semen Content and Its Effects in the Female Reproductive Tract.* JAmerican Journal of Physiology. Published.

*3. Keogan, Shawn, **Karl Siegert**, Brian Wigdahl, and Fred Krebs. *Factors in Human Seminal Fluid Reduce Epithelial Tight Junctions*. Submitted for Publication.

*4. Keogan, Shawn, **Karl Siegert**, Brian Wigdahl, and Fred Krebs. *Semen Induces Polarized Time- and Tissue-Dependent Release of Immunomodulatory Factors from Human Cervicovaginal Cell Lines.* Biology of Reproduction. Submitted for Publication.

*5. **Karl Siegert**, Brian Wigdahl, and Fred Krebs. *The Role Semen Plays in Altering Immune Cell Populations Found in the Female Reproductive Tract.* Submitted for Publication.

*6. **Karl Siegert**, Brian Wigdahl, and Fred Krebs. *Using an in vitro Model of HIV-1 Male-to-Female Transmission to Identify Key Aspects of the Bottleneck Hypothesis.* Submitted for Publication.

*7. **Karl Siegert**, Brian Wigdahl, and Fred Krebs. *Uncovering the Role Each Region of the Female Reproductive Tract Plays in the Bottleneck Hypothesis.* Submitted for Publication.

*8. **Karl Siegert**, Brian Wigdahl, and Fred Krebs. *The Biphasic Effect of Semen in the Female Reproductive Tract and its Role in HIV-1 Transmission from Male-to-Female.* Submitted for Publication.

Poster Presentations

1. **Siegert, K.,** Krebs, F. *Development of a More Physiological in vitro Model of Male-to-Female HIV-1 Transmission.* September 8, 2014. Discovery Day. Drexel University, Philadelphia, Pennsylvania.
2. **Siegert, K.,** Krebs, F. *Moving Toward a More Physiological Model of HIV-1 Transmission from Male-to-Female.* May 5, 2014. Infection and Immunity Conference. Drexel University, Philadelphia, Pennsylvania.
3. **Siegert, K.** Krebs, F. *in vitro Modeling the Female Reproductive Tract More Physiologically.* June 14, 2014. International Symposium on Molecular Medicine and Infectious Disease. Drexel University, Philadelphia, Pennsylvania.
4. **Siegert, K.** Krebs, F. *Modeling the HIV-1 Transmission Bottleneck.* October 8, 2016. Discovery Day. Drexel University, Philadelphia, Pennsylvania.

5. **Siegert, K.** Krebs, F. *Elucidating the Most Stringent Bottleneck in Male-to-Female HIV-1 Transmission.* November 30, 2015. Infection and Immunity Conference. University of Pennsylvania, Philadelphia, Pennsylvania.

RESEARCH PRESENTATIONS

2013 *The Effect of Aging on the Risk of Male-to-Female Transmission of HIV-1.* Student Seminar. Drexel University, Philadelphia, Pennsylvania

2014 *Moving toward a More Physiological in vitro Model of HIV-1 Male-to-Female Transmission.* Student Seminar. Drexel University, Philadelphia, Pennsylvania

2014 *in vitro Modeling of HIV-1 in the Female Reproductive Tract.* Student Seminar. Drexel University, Philadelphia, Pennsylvania

2015 *Modeling the HIV-1 Transmission Bottleneck.* Student Seminar. Drexel University, Philadelphia, Pennsylvania

2016 *Using an in vitro Model of HIV-1 Male-to-Female Transmission to Identify Key Aspects of the Bottleneck Hypothesis.* Dissertation Defense. Drexel University, Philadelphia, Pennsylvania

LEADERSHIP

Stonewall Sports League Philadelphia, PA 08/2011 – Present

- Captain for Intramural Sports
  - Kickball
  - Volleyball
  - Dodgeball

Tabu Lounge & Sports Bar Philadelphia, PA 01/2015 – 09/2015

- Hosted Quizzo one night a week
  - Created own questions
  - Emceed the event from start to finish
  - Reinvented the event for further use

Drexel University Philadelphia, PA 09/2013 – 06/2014

- President of the Biology Graduate Student Association
  - Official representative of the Graduate Student Body
  - Organized and oversaw general and executive board meetings
  - Worked directly with the other chairs to fulfill obligations
  - Establish and dissolve ad hoc committees